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Efeitos do genótipo e do ambiente na concentração de compostos benéficos para a saúde em quatro variedades de trigo duro

Genotypic and environmental effects on the concentration of healthy compounds of four durum wheat varieties

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Dissertation submitted to the University of Aveiro to fulfill the requirements to obtain the degree of Master of Biotechnology, specialization in Food Biotechnology, conducted under the experimental supervision of the Dr. Roberto Lo Scalzo, Research Responsible at Council for Agricultural Research and Economics – Food Technology Research Unit (Milan, Italy), and the supervision of the Dr. Ivonne Delgadillo, Professor at the Chemistry Department of the University of Aveiro (Aveiro, Portugal).

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I dedicate this work to those who remain in my heart.

In special, to Andrea.

jury

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palavras-chave

Trigo, trigo duro, amido resistente, fibra dietética, ácidos fenólicos, antioxidantes, benefícios para a saúde, efeito do ambiente de crescimento, efeito do genótipo.

resumo

O trigo é um dos cereais mais importantes na alimentação humana e um dos mais produzidos a nível mundial. No momento em que as alterações climáticas parecem ser cada vez mais importantes na produção de alimentos, foi questionado se as condições climáticas e o genótipo poderiam influenciar a produção de compostos benéficos para a saúde em variedades antigas e novas de trigo duro. O amido resistente e os ácidos fenólicos foram quantificados através de técnicas cromatográficas para avaliar os efeitos ambientais e genotípicos e para caracterizar quatro variedades de trigo duro cultivadas no sul de Itália. O ambiente influenciou a produção de amido resistente e de ácidos fenólicos, enquanto que o genótipo teve o maior impacto nestes. A produção de ácidos fenólicos tendeu a aumentar pelo efeito da estação invernal e do ano 2014 no período de enchimento dos grãos. Os ácidos ferúlico e sinápico foram os mais abundantes nestas variedades. As duas linhas genotípicas Etiopia novas foram as mais eficientes na produção de ácidos fenólicos e amido resistente, enquanto que o genótipo antigo Trinakria e o seu par geneticamente modificado mostraram ser ligeiramente menos produtivos. Pensa-se que os produtos alimentares à base de trigo com um conteúdo de amido resistente e ácidos fenólicos elevado conduzem a uma dieta mais rica em substâncias bioactivas que promovem a saúde humana.

keywords

Wheat, durum wheat, resistant starch, dietary fiber, phenolic acids, antioxidants, health benefits, effect of growth environment, effect of genotype.

abstract

Wheat is one of the most important grain in human diet and it is the most grown cereal crop wordwide. Nowadays since global climatic changes have become more important to food production, we asked whether climatic conditions and genotype would influence the production of healthy compounds on old and new varieties of durum wheat. Resistant starch and phenolic acids were quantified by HPLC techniques to evaluate the environmental and genotypic effects and to characterize four durum wheat species grown in South Italy. Environment had a strong impact on the production of resistant starch and phenolic acids, while genotype had the greastest effect on the same compounds. The production of phenolic acids tended to increase by the effect of winter sowing season and the year 2014 during the grain filling period. Ferulic and sinapic acid were the most abundant in the four varieties. The two new Ethiopian lines were more efficient on the production of phenolic acids and resistant starch, while the old genotype Trinakria and its genetic modified pair showed to be slightly less productive. Wheat based products higher in phenolic acids and resistant starch might lead to a diet richer in bioactive substances that promote health.

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Abbreviations

4-OHB, 4-hydroxybenzoic acid

AACC, American association of Cereal Chemists

AMG, Amyloglucosidase

AOAC, Association of Official Agricultural Chemists

A/X ratio, Ratio of arabinose to xylose

CAT, Catechin

CAF, Caffeic acid

p-COU, *p*-Coumaric acid

C.R.E.A., Council for Agriculture Research and Economics

C.R.E.A. – I.A.A., Council for Agriculture Research and Economics – Food Technology Research Unit

C.R.E.A.- C.E.R., Council for Agriculture Research and Economics – Cereal Research Center

Di-OHB, Di-hydroxybenzoic acids (meaning 3,4-di-hydroxybenzoic acid, 2,5-di-hydroxybenzoic acid, 3,5-di-hydroxybenzoic acid and 2,3-di-hydroxybenzoic acid)

EtOH, Ethanol

FAO, Food and Agriculture Organization of the United Nations

FER, Ferulic acid

GI, Glycemic index

GIT, Gastrointestinal tract

H_2O, Water

HPLC, High performance liquid chromatography

HPLC-DAD, High performance liquid chromatography - Diode array detection

HPLC-RI, High performance liquid chromatography – Refractive index

LPL, Lysophospholipids

NaOH, Sodium hydroxide

NRS, Non-resistant starch

PA, Phenolic acid

PAs, Phenolic acids

PRO, Protocatechuic acid

ROS, Reactive oxygen species

RS, Resistant starch

SIN, Sinapic acid

SPE, Solid Phase Extraction

SYR, Syringic acid

T, Temperature

T. aestivum, *Triticum aestivum*

T. durum, *Triticum durum*

TOT, Total

VAN, Vanillic acid

1. State of the Art

1.1. Introduction

Wheat (*Triticum* spp.) is one of the most important grain in human diet and it is the most grown cereal crop worldwide[1–3]. Starch is its major constituent polysaccharide of high nutritional value[3]. Currently, we use wheat as flour, a product from milling the wheat kernels, to generate an ample range of foods, such as bread, cookies, and pasta, which are the main source of food for hundreds of millions of people in the world.

According to FAO (Food and Agriculture Organization), in 2012, wheat was the fourth food product being produced in the world[4]. In ten years, between 1993 and 2013, the world production of wheat raised from 564,470,561,00 to 713,182,914.30 tonnes (see Figure 1)[5]. Wheat is part of most diets in the world, because it is adaptable to field production, easy to storage, a good nutritional source, and its flour has many food and technological applications[6].

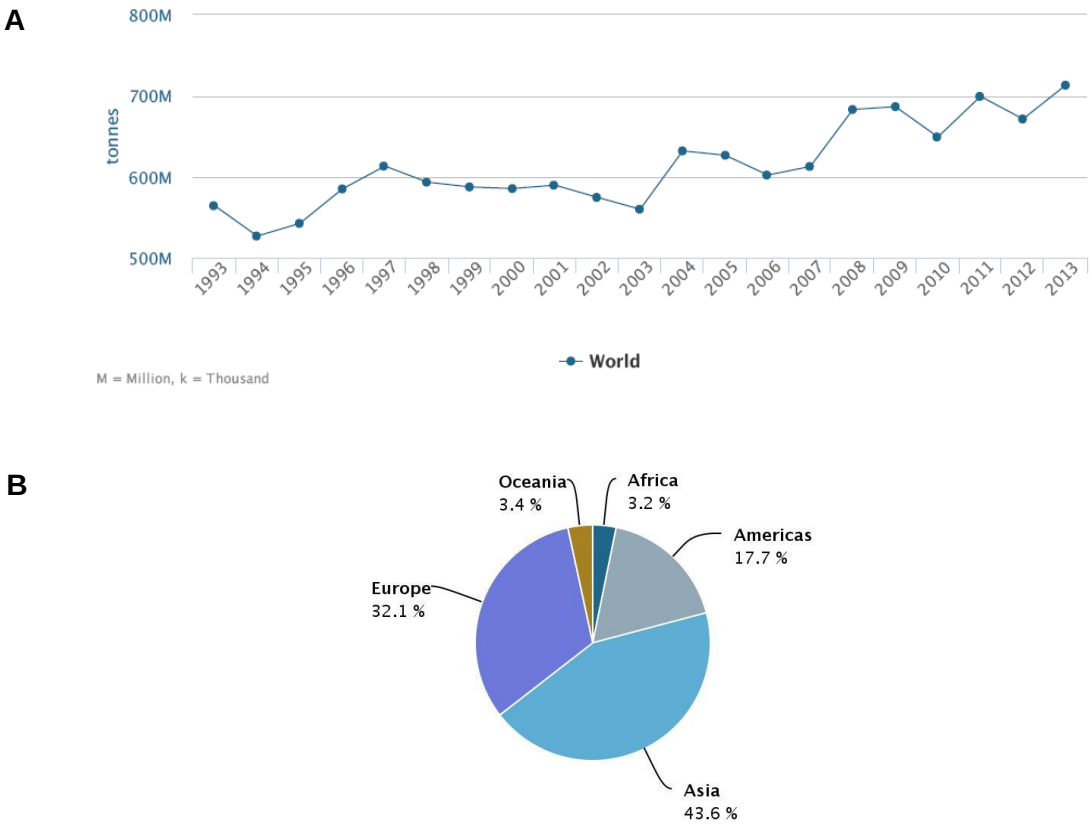


Figure 1: A) Wheat total production in the world, and B) Production share by region, from 1993 to 2013[7].

Carbohydrates and fibres are major nutrients of wheat, as well as other essential minor nutrients, including proteins, phenolics, enzymes, vitamin B, calcium, phosphorous, zinc, potassium, magnesium, niacin and most of the iron, riboflavin[6]. These compounds, located in specific structural parts of the grain, may suffer changes during the milling process to generate flour, however some processing is essential for palatability, sensorial qualities, safety and adequate nutrient bioavailability[8].

The constituents of the grain cell walls, especially the polysaccharides which are the major source of dietary fiber, and other minor components such as polyphenols, are very important for human nutrition.

A whole-wheat product implies that all the three parts of the wheat kernel (bran, endosperm, germ) are incorporated[9]. Subsequently, whole grain foods that preserve most of the pericarp (bran), aleurone (bran) and germ provide more macronutrients and significant nutrients when compared to the white refined flour, which contain mainly the starchy endosperm[8,10]. The members of the American Association of Cereal Chemists (AACC) committee created a definition of whole grain that aim to benefit both processors and consumers, accepted and approved by the Board of Directors in 1999[11]:

"Whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components — the starchy endosperm, germ and bran — are present in the same relative proportions as they exist in the intact caryopsis."

Len Marquart, an AACC member (General Mills, Minneapolis), adds "The benefit of keeping the whole grain components in proportion is that it provides a balance of nutrients and non-nutrients that may work together to reduce the risk of chronic disease". In fact, Dykes and Rooney (2007) report that all these health-promoting compounds present in whole-wheat grains may act in synergy to reduce the incidence of some chronic diseases, such as deficient transit time, cardiovascular disease, type-2 diabetes and gastrointestinal cancer[8,10,12]. This fact is rather important since morbidity and mortality, caused by non-infectious

diseases in affluent, developed economies, are closely related with human diet. An accepted strategy to lower the socio-economic impact of these conditions is to adapt diet and lifestyle, which are the main risk factors of diet-related diseases, as well as implementing a disease prevention system.

Wheat grain (*Triticum* spp.) contains about 70% starch and 12-18% protein[3]. Protein content may vary greatly in wheat (from 6% up to nearly 20%) as it is determined by wheat class, soil fertility and environmental conditions during the growing season[13]. Durum wheat (*Triticum durum*) is particular for its higher protein content when compared to soft wheat varieties[14]. The nutritional profile of durum wheat versus soft wheat is shown on Table 1.

Table 1: Nutritional profile of a durum wheat variety alongside to a soft wheat variety from Sicily[14].

Components	**Durum wheat / 100g**	**Soft wheat / 100g**
Water	14.2 g	14.2 g
Protein	12.5 g	11.0 g
Lipids	1.0 g	0.7 g
Carbohydrates	75.2 g	77.3 g
Starch	66.7 g	68.7 g
Soluble sugars	1.8 g	1.7 g
Fibre	3.5 g	2.4 g
Sodium	2 mg	3 mg
Potassium	140 mg	126 mg
Iron	0.9 mg	0.7 mg
Calcium	18 mg	17 mg
Phosforous	160 mg	76 mg
Thiamine	0.25 mg	0.10 mg
Riboflavin	0.04 mg	0.03 mg
Niacin	1.20 mg	1.00 mg

In the production of spaghetti and other pasta products it is typically used durum wheat semolina. Semolina is obtained by repeated grinding and sieving of

the durum, which results in granular endosperm and small amounts of bran powder[9]. In whole wheat semolina, the bran remains in the product, and contributes with rich nutrients[8]. Wheat variety, climatic conditions, irrigation, and fertilization have significant effects on crop size, wheat characteristics and consequently on semolina quality[9]. Winter wheat grains have shown to provide for the highest yields, especially from highly productive varieties, in the presence of adequate nutrients on fertile soils with adequate water supply[3].

1.2. Taxonomy of durum wheat

Wheat belongs to the class of *Monokotyledonae* plants and to the family Poaceae[15]. *Triticum* is the genus name for wheat[6].

Triticum aestivum is the species name for common wheat (hexaploid, genomes A, B, and D), the most cultivated wheat worldwide (> 90%) commonly used for bread production[6].

Durum wheat was described by René Louiche Desfontaines (Desf.), who gave it the species name of *Triticum durum* (tetraploid, genomes A and B)[6]. *T. durum* has been derived from the natural hybridization of *Triticum monococcum* (A genome) and the ancestral *Aegilops speltoides* (B genome)[6]. Now, *T. durum* is predominantly used for pasta production[6].

1.3. Primary and secondary plant metabolites

Plant metabolites comprise more than 100,000 different compounds, divided into primary and secondary metabolites[16]. Primary metabolites (macronutrients), i.e. carbohydrates, proteins and fats, are essential nutrients for the human diet providing energy to the cells. Secondary metabolites (also known as *phyto*-chemicals) comprise a wide range of low molecular weight substances chemically different and ubiquitous in plants[16]. These metabolites do not seem to be directly essential to humans, but do often cover important physiological roles[16]. Phytochemicals, e.g. phenolic compounds are involved in interactions with biotic and abiotic environments.

1.3.1. Carbohydrates in whole wheat grains

The carbohydrate constituents of cereal grains are derived from structures generated by photosynthesis, in chlorophyll-containing cells of leaves, stems, and outer integuments of the developing grain. They are subsequently translocated from these tissues through the vascular system to the ripening grain[17].

The mature grain of common wheat (*T. aestivum*) consists of 85% (w/w) carbohydrates, approximately 80% of which is starch (only in the endosperm); ~7% low molecular mass mono-, di-, and oligosaccharides (in the aleurone, starchy endosperm, and tissues of the embryonic axis), and fructans (in the starchy endosperm and embryonic axis); and ~12% cell wall polysaccharides (in all tissues)[17]. Starch and fructans are storage polymeric structures of carbohydrate, which are mobilized during the germination of the grain as carbon and energy sources for the developing embryo[17].

Low molecular mass carbohydrates from the aleurone, endosperm, and embryo of the mature grain, are present in trace amounts, and they can be extracted with water or 80% (w/v) ethanol[17]. These fraction of carbohydrates comprehend the reducing aldohexose monosaccharides, D-glucose and D-fructose, and little amounts of their phosphorylated forms (intermediates in carbohydrate metabolism)[17].

Sucrose is a non-reducing disaccharide constituted of D-glucopyranosyl and D-fructofuranosyl residues, and also raffinose and small amounts of 1-kestose and 6-kestose[18]. Maltose and melibiose, both disaccharides, are found in slight amounts[19]. The relative amounts of low molecular mass carbohydrates in aleurone cells are: sucrose (42%), raffinose (31%), neo-kestose (20%), and fructosyl raffinose (6%); whereas monosaccharides, maltose, and higher oligosaccharides are not present[20].

Cereal fructans are of the inulin type, and contain chains of β-D-fructofuranosyl residues (2→1)-β-linked to the fructosyl residue of sucrose, with some branching through (2→6)-β linkages[17]. Fructans make up for 1.3-2.5% (w/w) of mature grain[21–23], and they are extractable in water and 80% ethanol[17].

Carbon metabolism of the grain is a key physiological process which determines crop growth, yield and quality, and that is very sensitive to abiotic stresses (e.g. drought and heat)[2].

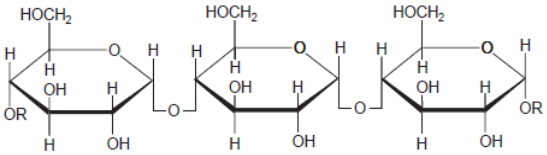
1.3.1.1. Starch

Starch is the major polysaccharide found in almost every seeds that are grown and used for human consumption[8]. Before, even though recognized as the main component (60-70% dry weight)[2] of the wheat grain, starch was considered “inert” as only an important source of energy, and not significant for the quality of wheat compared to the protein fraction[17]. However, the interest on starch properties has been evident because of its influence on the end-product quality, and the importance of its digestibility for human nutrition.

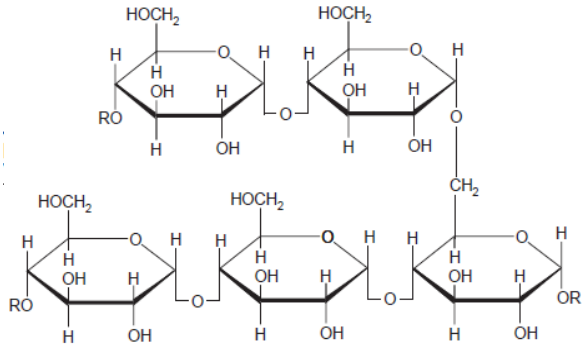
Starch of the wheat grain is composed of amylose (25-30%) and amylopectin (70-75%), both polymers of glucose[2]. Amylose is a linear molecule of α-(1-4)-D-glucopyranosyl units, though some molecules are slightly branched by occasional α-(1→6) branch points (Figure 2)[24]. This molecule is susceptible to the β-amylase enzymatic hydrolysis, which breaks the (1→4) bonds from the non-reducing end of the chain, releasing units of maltose, and remaining the (1→6) linkages that are named β-limit dextrin. β-Limit dextrins of branched amyloses also have properties (e.g. iodine binding capacity) close to the properties of the respective original amyloses, and very much apart from those of amylopectin[24]. In addition, amylose chains in solution do not behave significantly different from the strictly linear chains[24].

Amylopectin is the highly-branched component of starch, formed by hundreds of short α-(1-4)-D-glucopyranosyl chains with 5-6% of (1→6) bonds at the branch points (Figure 2)[2,24]. The debranching enzymes, isoamylase and pullulanase, specifically hydrolyze the branch linkages and generate short linear chains[24].

Despite the high molecular weight of amylopectins, they have low intrinsic viscosity conferred by its own branched character[24].



A) Amylose



B) Amylopectin

Figure 2: Structure of amylose and amylopectin, the two components that made up starch. A) Amylose: α-(1→4)-glucan; average number of residues (n) = ca. 1000. A few occasional moderately long chains may occur through α-(1→6) linkages. B) Amylopectin: α-(1→4)-glucan containing α-(1→6) branching points; n of branched chains vary according to the botanical origin[93]. Image adapted from Slattery *et al.* (2000)[94].

The physicochemical and functional features of starch are mostly contributed by amylopectin[25]. A higher content of amylose decreases starch hydrolysis and increases the amount of resistant starch because of the physicochemical features of amylose. Soluble dietary fiber reduces the rate of glucose release in foods through increased viscosity and/or slowing down gastric emptying[26].

Lipids (mostly triglycerides), a minor component of starch, are generally present in cereal starch granules, and a content in the range of 0.8-1.2% can be found in wheat grains[24]. Wheat starches contain mainly lysophospholipids (LPL), being lysophosphatidylcholine the major lipid found in wheat, together with palmitic and linolenic acids[24]. Both LPL and amylose contents increase with the maturity of the wheat grain[24]. Monoacyl lipids, associated with lipolysis, induce the formation of amylose-lipid complexes during gelatinization, and thus will restrict swelling, dispersion of the starch granules and solubilization of amylose[24]. Phosphorous in cereal starches occur mainly in the form of phospholipids[24].

The main surface components of granular starch are proteins (e.g. friabilin), enzymes, amino acids and nucleic acids[24]. The starchy endosperm contains the principal gluten storage proteins, gliadins and glutenins, and small amounts of globulin (triticin) storage proteins[27]. Gluten quality and quantity influence dough

and bread characteristics by absorbing and retaining water during the baking process[9]. The amount and strength of gluten in durum wheat are directly related to pasta quality[9].

The important role of starch in the production and quality of baked foods is because it is a source of fermentable sugars that feed yeast[13]. However, it is also the responsible for some negative changes of baked foods through recrystallization or retrogradation[13].

1.3.1.2. Resistant starch

Starch digestibility is taking an important role for its opportunity of health delivery. Slowing starch digestion leads to a lower glycemic response, whereas reducing the total amylolysis at small intestine makes the amount of resistant starch (RS) increase[8]. Resistant starch is an important component of dietary fibre, as the portion of starch that is resistant to enzymatic digestion at the small intestine, which breaks down and ferments at the large bowel[28].

Table 2: Types and food sources of resistant starch[a].

Types of RS	Feature	Sources
RS_1	Physically inaccessible starch	Whole or partially intact grains and legumes
RS_2	Starch in its natural form	Ungelatinised granular starch; e.g. raw potatoes, unripe bananas, some legumes and high amylose corn
RS_3	Retrograded starch	Cooked and cooled starch-containing foods, bread, cornflakes and retrograded high amylose corn
RS_4	Chemically modified resistant starches	Industrially processed food ingredients, e.g. breads and cakes

[a] Adapted from Sajilata *et al. (*2006) and from Rahman *et al.* (2007).
RS, resistant starch.

High amylose content starch is associated with increased resistant starch, representing an advantage since amylose is digested more gradually than amylopectin[28–30]. This characteristic of starch helps to maintain lower levels of

blood glucose and insulin after meals[30]. Foods high in resistant starch content have reduced glycemic indices, important in obesity and diabetes prevention, and enhance bowel movements[28,31].

Structure of resistant starch can be divided in three types: RS_1 is physically inaccessible to digestive enzymes; RS_2 occurs in its raw form, such as ungelatinized starch; RS_3 is the retrograded starch; RS_4 are chemically modified starches[32–34]. Structure and sources of resistant starches are demonstrated in Table 2[32,33].

1.3.1.3. Digestion in human gastrointestinal tract

Digestion is the process of breaking down the food structures and matrices, where the nutrients are kept, so that nutrients can be released and absorbed through the walls of the gastrointestinal tract (GIT)[25]. The human GIT comprehends the oral cavity, the esophagus, the stomach, the small intestine (includes duodenum, jejunum, and ileum), the large intestine (ascending, transverse, and descending colon), rectum, and anus[25]. When a starchy food is ingested, the α-amylase, a salivary enzyme, partially hydrolyses amylose and amylopectin structures, consequently decreasing the viscosity of these foods[25]. The digestion and the release of nutrients, and their conversion into an absorbable form, occur mainly in the small intestine[25]. Carbohydrates are digested by amylase and glucosidase into monosaccharides. The digestion of disaccharides and some oligosaccharides is carried out by the enzymes of the small intestinal brush border[25]. The undigested carbohydrates and proteins are thereafter fermented by the colon microbiota[35], generating short-chain fatty acids, in particular acetate, propionate, and butyrate, which are subsequently absorbed in the colon[25].

1.3.1.4. Cell walls composition of wheat grain

Cereal grain cell walls, including their polymeric constituents, have a wide diversity of structure and organization[36]. The content, structure, and composition of the cell wall polysaccharides in the mature grain vary according to cultivars[36] and tissue[37]. The composition of the embryo of wheat grains remains unclear[38].

The cell walls and their constituent polysaccharides are the major elements of dietary fiber, and they are associated to the main beneficial effects of the cereal grain in human health[36].

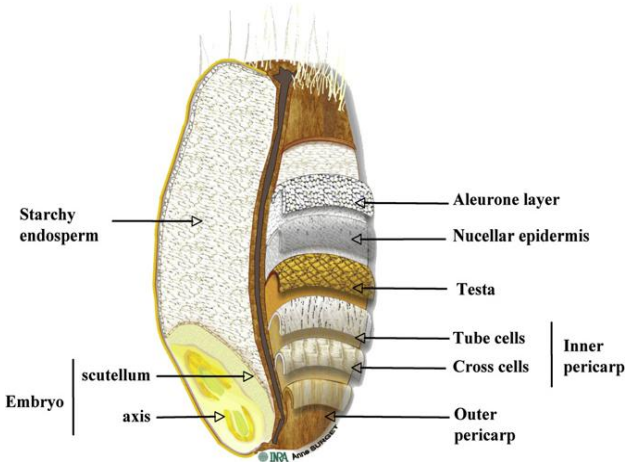


Figure 3: Grain anatomy of wheat. Adapted from Surget and Barron (2005).

1.3.1.4.1. Endosperm

Arabinoxylans and β-D-glucans are the major polymers in the cell walls of the endosperm of wheat grains (see Figure 3)[36]. Wheat is more rich in arabinoxylans than of β-glucan[36]. Other polymers have also been found but in lower amounts, such as cellulose (2-4%), glucomannan (2-7%), structural proteins and proteoglycans (e.g. arabinogalactanpeptides)[39]. Structural proteins integrated in wheat cell walls contribute to the physical properties of the wall[36].

Arabinoxylans in the endosperm of wheat grain contain arabinose, xylose, and a small amount of ferulic acid[36]. The general structure of the arabinoxylans found in wheat flour is shown on Figure 4: and Figure 4.

Arabinoxylans are present in water-extractable and water un-extractable fractions[36]. Of the total arabinoxylan content in wheat flour (endosperm) approximately ¼ are water-extractable arabinoxylans[36]. The remaining arabinoxylans are water un-extractable arabinoxylans, the major arabinoxylans of the endosperm cell walls, which structure is very similar to the water extractable ones, except for the average molecular weight and the ratio of arabinose to xylose

(A/X ratio) that are somewhat higher for water un-extractable arabinoxylans[40]. In wheat, the extent of ferulic acid linked to these arabinoxylans is low (about 1%)[36].

Cell walls located close to the germ are enriched in β-glucan[36].

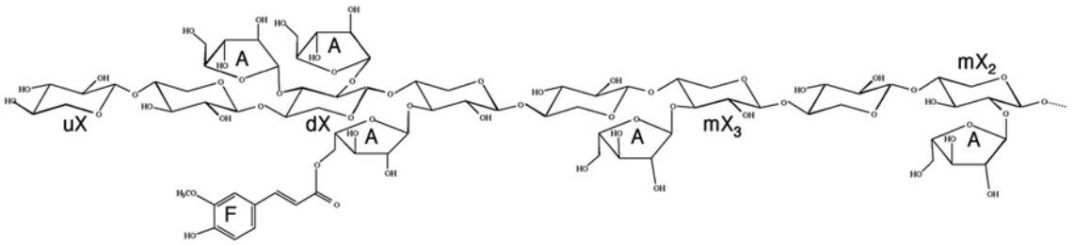


Figure 5: Main structure of arabinoxylans from endosperm cell walls of cereal grains. A: arabinose, X: xylose, F: ferulic acid, uX: unsubstituted xylose, dX: di-substituted xylose, mX_3: O-3 mono-substituted xylose, mX_2: O-2 mono-substituted xylose (rare in wheat endosperm arabinoxylans). Adapted from Saulnier *et al.* (2012)[36].

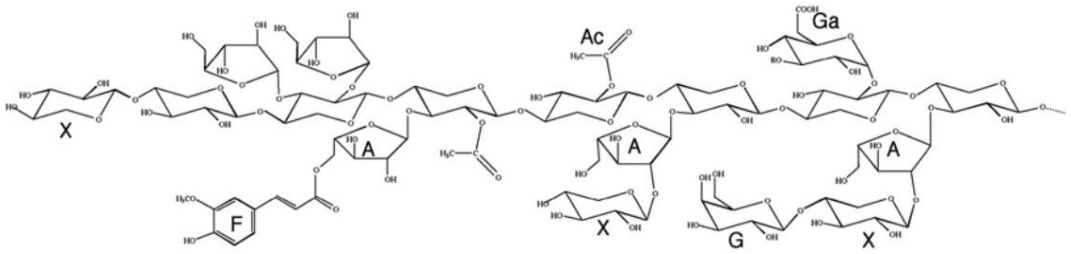


Figure 4: Main structure of arabinoxylans from outer tissues cell walls of cereal grains. A: arabinose, X: xylose, G: galactose, Ga: glucuronic acid, F: ferulic acid, uX: unsubstituted xylose, dX: di-substituted xylose, mX_3: O-3 mono-substituted xylose, mX_2: O-2 mono-substituted xylose (rare in wheat endosperm arabinoxylans). Adapted from Saulnier *et al.* (2012)[36].

Arabinoxylans have different structures according to the cell localization, for instance in prismatic cells, arabinoxylans occur more highly substituted than in central cells (see Figure 6)[36]. This spatial variation may be the reason for the chemical heterogeneity observed amongst arabinoxylans isolated from wheat flours, suggesting a strong regulation of the biosynthetic system[40]. In fact, structural studies of water-extractable arabinoxylans indicated that the arabinose to xylose (A/X) ratio is strongly positive correlated to the proportion of di-substituted xyloses and negative correlated to the proportion of un-substituted

xyloses, while the proportion of mono-substituted xyloses is independent of A/X ratio[40]. Structural heterogeneity of arabinoxylans is not only restricted to arabinose substitution, but also to bound ferulic acid content[40]. According to Dervilly *et al.* (2000)[41], water-extractable arabinoxylans with low A/X ratio contain higher content of ferulic acid than water-extractable arabinoxylans with high A/X ratio, suggesting that feruloylated-arabinose residues may be mainly found as a single side-chain of xylose residues (mono-substituted xylose).

Feruloylated arabinoxylans are the major non-starch polysaccharides from the cereal cell walls, which represent a significant portion of human dietary fibre intake, adding nutritional benefits as soluble and insoluble fibre[42]. The presence of ferulic acid bounded to arabinoxylans confers them antioxidant properties[42].

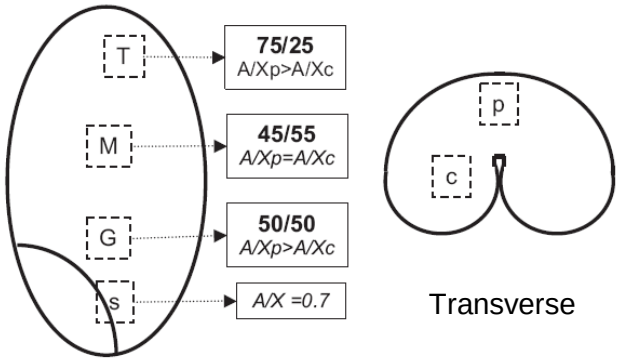


Figure 6: Variation in polymer structure and composition within wheat endosperm in mature grain. p: prismatic cells, c: central cells, T: top area, M: median area, G: germ area, s: scutellum and transfer cells; A/X: arabinose to xylose ratio, A/Xp: arabinose to xylose ratio in prismatic cells, A/Xc: arabinose to xylose ratio in central cells. Adapted from Saulnier *et al.* (2012)[36]

1.3.1.4.2. Aleurone

Aleurone cells surround the starchy endosperm, as a peripheral layer, except in the crease region where a different type of cells appear – transfer cells, which are modified aleurone cells[36]. Some authors (e.g. Thompson *et al.* (2001)[43]) believe that the transfer cells promote solute transfer between tissue compartments, and participate in the solute uptake of grain.

The wheat aleurone cell walls are mainly composed of arabinoxylans and β-glucan, in addition, low amounts of cellulose and lignins are present[38]. Lignin is a polymeric structure that involve three types of hydroxycinnamyl alcohols (monolignols): *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which differ only on the degree of methoxylation; however, composition of lignin in wheat grains is not well-known[36].

In the aleurone layer, the ratio β-glucan/arabinoxylans is higher (40/60 g/g β-glucan/arabinoxylans) than in the cell walls of the starchy endosperm[44]. The arabinoxylans of aleurone are structurally similar to the arabinoxylans of the starchy endosperm, however in the aleurone layer they are not water-extractable, have a lower A/X ratio, and are greatly esterified, comparatively to those of the starchy endosperm[44]. The *p*-coumaric acid, an hydroxycinnamic phenolic compound, accounts for 10% of the bound phenolic acids in the aleurone layer[38], although it is not recognized if it is linked to arabinoxylans or to other aleurone cell wall component[36].

The aleurone cell walls contain 4-5% (w/w) protein associated with cellulosic glucan, glucomannan and highly substituted arabinoxylans[36]. Protein-polysaccharide cross-linking through dimerisation of tyrosine-hydroxycinnamic acid was suggested by Rhodes and Stone (2002)[45], and protein insolubility may be partly due to the cross-linking by tyrosine-tyrosine bridges.

1.3.1.4.3. Outer layers

The outer layers of the kernel play a protective role in the grain[36]. These outer layers consist of (from the outside to the inside) the outer pericarp, the inner pericarp (that contain cross cells and tube cells), the pigmented seed coat (testa), the nucellar epidermis, and the nucellus (Figure 3)[36]. In these tissues, the cell walls are thick, hydrophobic and formed predominantly by cellulose and complex xylans, as well as lignin found generally in significant amounts (10-12%)[46].

Glucuronic acid, or its 4-O-methyl ether, and galactose are present in arabinoxylans of the outer layers, described also as glucuronoarabinoxylans or heteroxylans[36].

The outer tissues of the kernel contain high contents of hydroxycinnamic acids (mainly *p*-coumaric, ferulic and sinapic)[36] and dihydrodiferulic acid[38].

1.3.1.4.4. Architecture of the cell walls

Cereal grain cell walls of endosperm are mainly constituted of arabinoxylans and mixed-linked β-glucan, whereas in the outer tissues are arabinoxylans, cellulose, and variable amounts of lignin. The interactions of cellulose-arabinoxylans are thought to occur through cellulose absorption of xylan-xylan interactions, which are induced by high levels of unsubstituted xylosyl residues[36]. The highly substituted arabinoxylans at the endosperm/outer pericarp cell walls do not favor the interaction with cellulose[36].

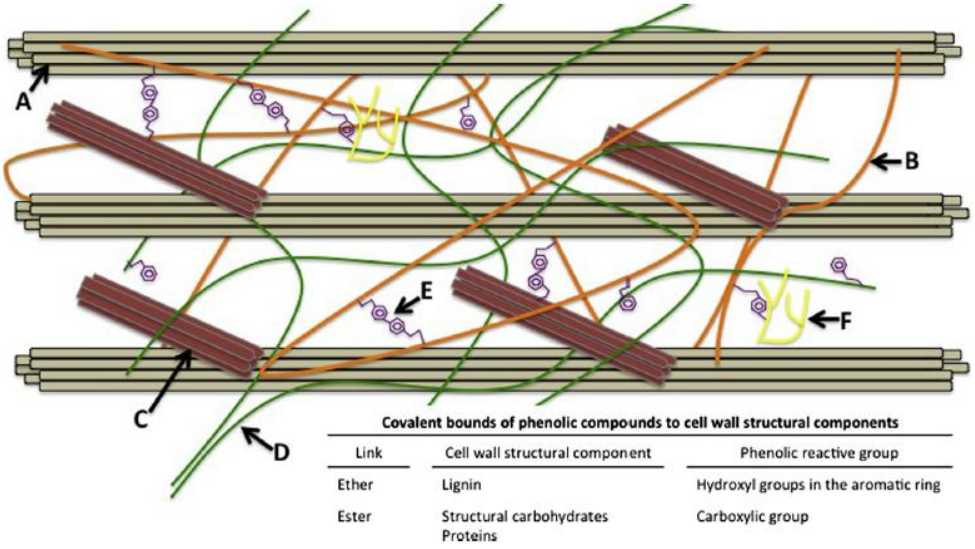


Figure 7: Structure of plant's primary cell wall, representing structural components cross-linked with phenolic compounds. (A) Cellulose, (B) Hemicellulose, (C) Structural proteins, (D) Pectin, (E) Phenolic acids, (F) Lignin. Adapted from Acosta-Estrada et al. (2014)[49].

Covalent cross-linking of arabinoxylan chains through ferulic acid dehydrodimers and trimers may be the main factor to make wall assembly of cereal grains possible[47]. Hydroxycinnamic acids are esterified or etherified to the lignin surface[48], and it is probable that ferulic acid etherified to lignin occurs also esterified to arabinoxylans[47]. It has been suggested direct ester linkages among uronic acid (glucuronoxylans) and hydroxyl groups of lignin surfaces, as well as

ether links between arabinoxylans and lignin by e.g. primary hydroxyl of arabinose side chains[47].

The highly structural and compositional diversity of the cell wall polysaccharides of the outer layers and endosperm reflects the physiological functions of the different tissues (Figure 7).

1.3.2. Phenolic compounds in wheat whole grain

Whole grains contain unique phytochemicals that complement those in other plant-based foods, like fruits and vegetables[50]. Phytochemicals are important for the structure of the grain and they also play a role on their defense[50]. They act as antioxidants and free-radical scavengers (e.g. ROS: superoxide anion, hydroxyl radicals, and peroxy radicals) to combat oxidative stress and to help maintain the balance of oxidants-antioxidants[51]. Their additive and synergistic effects may be responsible for the acclaimed beneficial effects on human health, for example the reduction of chronic disease incidence[52]. Phenolic compounds, lignans, carotenoids, vitamin E, and phytosterols are main groups of phytochemicals present in whole grains.

1.3.2.1. Phenolic compounds: Phenolic acids

Phenolic compounds are essential for the growth and reproduction of the plants, in defense mechanisms against pathogens, parasites and predators, and contribute to the color of the plants[50]. Phenolic compounds are produced by the shikimic pathway occurring in the plastids and they are derived from the aromatic acids phenylalanine or tyrosine[53,54].

Phenolic compounds are structures which have one or more aromatic rings, with one or more hydroxyl substituent groups[52]. They occur mostly as glycosides bounded to a variety of sugar moieties in the pericarp of cereal grains, making them water-soluble compounds[10,15]. Alternatively, they occur as complexes linked to other structures such as organic acids, amines, lipids, carbohydrates, or other phenols[50].

The most commonly found phenolic compounds in whole grains are phenolic acids (Figure 8) and flavonoids, and in less amount anthocyanins, stilbenes, coumarins, and tannins[10,50,52].

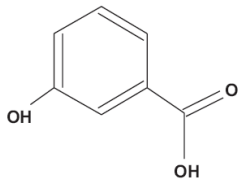
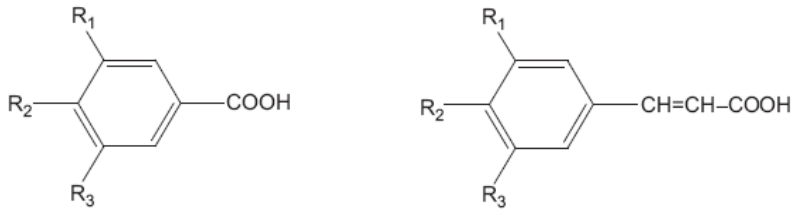


Figure 8: Basic structure of phenolic acids, one of the most commonly found group of phenolics in whole grains. Image from Liu (2007)[50].

Phenolic acids can be subdivided in two main groups: hydroxybenzoic acid and hydroxycinnamic acid derivatives (Figure 9)[50]. Hydroxybenzoic acid derivatives comprise *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids[50]. Commonly present in wheat are vanillic and syringic acids[15]. In general, they exist in the bound form as part of complex structures, such as lignins, and hydrolysable tannins[50]. Hydroxycinnamic acid derivatives comprise *p*-coumaric, caffeic, ferulic, and sinapic acids[50]. They as well are mainly present in the bound form, linked to the cell wall structural components, such as to cellulose, lignin, and proteins, by ester bonds[50].



a) **b)**

Figure 9: **a) Benzoic acid derivatives:** Benzoic acid (R_1= H; R_2= H; R_3= H), *p*-Hydroxybenzoic acid (R_1= H; R_2= OH; R_3= H), Protocatechuic acid (R_1= H; R_2= OH; R_3= OH), Vanillic acid (R_1= CH_3O; R_2= OH; R_3= H), Syringic acid (R_1= CH_3O; R_2= OH; R_3= CH_3O), Gallic acid (R_1= OH; R_2= OH; R_3= OH). **b) Cinnamic acid derivatives:** Cinnamic acid (R_1= H; R_2= H; R_3= H), *p*-Coumaric acid (R_1= H; R_2= OH; R_3= H), Caffeic acid (R_1= OH; R_2= OH; R_3= H), Ferulic acid (R_1= CH_3O; R_2= OH; R_3= H), Sinapic acid (R_1= CH_3O; R_2= OH; R_3= CH_3O). Images from Liu (2007)[50].

Ferulic acids are easily found in the cell walls of the wheat bran (esterified to hemicellulose)[50]. They occur primarily in the seeds and leaves of plants, and appear mainly conjugated covalently to mono- and disaccharides, polysaccharides of the cell wall, glycoproteins, polyamines, lignin, and insoluble carbohydrate biopolymers[50]. Industry processes of foods, like thermal processing, pasteurization, fermentation, and freezing, induce the release of these bound phenolic acids[55].

Ferulic, caffeic, *p*-coumaric, syringic, and vanillic acids are common phenolic acids present in whole grains, and ferulic is one of the most commonly found[56].

The cell walls of aleurone, pericarp and embryo of various grains are rich in ferulic acid, on the contrary to the starchy endosperm where it occurs in trace amounts[57]. Ferulic acid and other phenolic acids provide both physical and chemical barriers to wheat seed through cross-linking carbohydrates, antioxidant power (to combat destructive radicals), and astringency (to combat consumption by insects and animals)[58,59].

In whole grains, ferulic acid may occur in the free, soluble-conjugated, and bound forms[50]. Bound ferulic acid was predominantly found (>93%) over the free and soluble-conjugated forms in wheat, with a ratio of free, soluble-conjugated, and bound ferulic acid of 0,1:1:100[51]. The presence of ferulic acid in wheat whole grains varies among varieties and cultivars in mature grains[50]. Adom *et al.* (2003)[60] observed significant differences, some of up to two fold, on the total ferulic acid content of 11 wheat varieties, and that this compound existed mostly in the bound form (>97%) in all varieties. Additionally, studies of Yu *et al.* (2004)[61] showed that flours from the same wheat variety grown at different locations may differ significantly in their radical scavenging activity, suggesting that growing conditions might have significant influence on the antioxidant properties of wheat flours.

The pigmentation intensity of *T. durum* is determined by genetic factors, and it is strongly influenced by environmental conditions, in particular light intensity and temperature[1]. When wheat buds are strongly illuminated they may become more or less colored according to the variety, because of the formation of anthocyanins which occurs in different organs of the species[1].

1.4. Wheat and human health

1.4.1. Resistant starch and dietary fiber

1.4.1.1. Definition and properties

The first dietary fiber concept emphasized the plant cell-wall constituents as the main source of indigestible material, but after that resistant starch and oligosaccharides emerged as important substrates of fermentation for the large intestine microflora[62]. A number of beneficial effects of dietary fiber are regarded to its indigestibility in the small intestine, and to its action at the large intestine[62].

The physiological properties of food carbohydrates are the key to understand their nutritional effects on human digestive system. Some of the most important properties considered are the following:

- The absorption level in the small intestine determining the amount of carbohydrate used as a substrate by the body cells, and the amount used as a fermentation substrate in the large bowel microflora; and the absorption rate in the small intestine as a result for the blood glucose (glycemic index) and consequently for the metabolic response (e.g. insulin response)[62].
- The extent and rate of colonic fermentation; and the nature and proportions of fermentation products, principally the short chain fatty acids acetate, propionate and butyrate[62]. Acetate and propionate are absorbed, producing effects on lipid and possibly on carbohydrate metabolism[62]. Butyrate acts specifically as a main source of energy for the colonic epithelial cells, adding anti-tumor properties[62]. Overall, they also contribute to lower the pH of the large intestinal area, decreasing the formation and the solubility of co-carcinogenic bile salt derivatives[62].

The physiological properties of dietary fiber regarding hypoglycemic and hypolipidemic properties, among others, are more related to physical features, like solubility and viscosity, than to monomeric composition of dietary fiber constituents[62].

Some oligo- and polysaccharides are indigestible at the small intestine, such as resistant starch, non-starch polysaccharides and oligosaccharides[62].

Starch, a polysaccharide, is the quantitatively most important digestible carbohydrate in diets[62]. Starch has been regarded more nutritionally superior than low-molecular weight carbohydrates, because this polysaccharide is slowly digested and absorbed[62]. Dietary polysaccharides can be divided into starch (α-glucan), and non-starch polysaccharides (NSP)[62]. Dietary NSP, mostly found in plant cell wall structures, include cellulose (linear β-glucan), and a variety of heteropolysaccharides with no α-glucosidic bonds[62].

Resistant starch is the starch which resist the upper intestinal digestion and enter the lower intestine to be fermented by the microflora in the colon[50]. Physically trapped starches are trapped within food matrices, and thus have little interaction with the digestive enzymes in the small intestine[50]. They are usually found in whole and partly ground grains, seeds, and legumes; and its concentration and distribution depends on the food processing technique[50]. Resistant starch granules possess crystalline regions that are less susceptible to digestion through acid or α-amylase enzymes[50]. Retrograded high-amylose starch, which retrogrades faster than high-amylopectin starch, may resist to dispersion in water and digestion by α-amylase[50]. Resistant starch is able to improve glycemic response and colon health, offer low calorie intake, and also modulate fat metabolism[50].

β-Glucan is a group of linear polymers, comprised by units of glucose connected through 70% of β-(1→4) and 30% of β-(1→3)-linkages[50]. The β-(1→3) linkages interrupting the β-(1→4) linkages make β-glucan more flexible, soluble and viscous, when compared to cellulose made of only β-(1→4) linkages[50]. β-Glucan is frequently found in the cell walls of many grains, including wheat, barley, and oats[50]. In addition, it has been associated to the delaying of gastric emptying, making time for the dietary fiber to be more gradually absorbed.

1.4.1.2. Potential health benefits

Whole grains are a rich source of dietary fiber, which have been recognized as an essential element of a healthy diet. Dietary fiber in whole wheat grain is mainly present in the bran layers[12,13]. According to the United States Department of

Agriculture and Health Canada, the total dietary fiber content of wheat ranges from 11-12.7%, and on wheat flour the fiber content is as low as 2.0-2.5%[13].

Rui Hai Liu gives a general view of the physiological effects observed by the consumption of whole grain dietary fibre, which are due to binding and eliminating cholesterol, binding bile acids, modulating hormonal activity, stimulating immune system, facilitating toxicant transit in the digestive tract, production of short chain fatty acids in the colon, diluting gut substances, lowering caloric content and glycemic index of foods, enhancing insulin response, providing bulk in foods, and scavenging free radicals[50].

High fibre diets are important for the prevention and management of certain chronic diseases, such as type-2 diabetes, cardiovascular disease, and certain cancers.

Viscous fibres account for the majority of the benefits of dietary fibres[63]. The viscosity of these fibres slows digestion of nutrients through the prevention of bulk diffusion of foods across the intestinal lumen[63]. For instance, β-Glucan most important biological effects are the lowering of blood cholesterol levels, control of blood sugar and boost the immune system[50], when intake is adequate. These biological results are probably due to its high viscosity nature as a soluble fibre, once this feature makes it able to bind cholesterol and bile acids, helping in their elimination from the body[50].

Additional effects may come from dietary fibre intake on the basis of a low glycemic index (GI) diet[63]. The GI measures the change in blood postprandial glucose response after consuming a food product[64]. This concept appeared after the findings that different carbohydrate foods induce different glycemic response[62]. It seems that low GI (<65) foods are those that cause low postprandial glucose responses – based on the bread scale – which include pumpernickel bread, pasta, legumes, nuts, and parboiled rice[63]. Medium GI foods (65-89) include all-bran, oatmeal, sweet potatoes, and yam; whereas, high GI foods (>90) are most breads and breakfast cereals[63]. The reduction of nutrient absorption levels by viscous fibres lowers postprandial glucose and thus, insulin responses, which presents significant implications in the prevention and management of insulin-resistance and type-2 diabetes[63].

1.4.2. Phenolic compounds as antioxidants

Halliwell and Gutteridge (2007) defined the term antioxidant in a general and simplest way, as “any substance that delays, prevents or removes oxidative damage to a target molecule”[65]. Oxygen is an oxidant with affinity for electrons and hydrogen, important in the electron transport chain to generate metabolic water[66]. However, when dioxygen or its reduced derivatives randomly and uncontrollably oxidize other biological molecules, for example the lipids of the membranes, the biological damage occurs[66].

Oxygen and the reactive species of oxygen are initially produced by a single electron reduction of oxygen[66]. The intermediate steps of the molecular oxygen (dioxygen) reduction include the formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) (Figure 10)[67,68]. These are reactive species capable of oxidizing organic molecules, particularly unsaturated hydrocarbons[66]. Oxygen radicals may also occur as alkyl or peroxyl radicals[67]. They might generate by means of lipid peroxidation, or radiation by exposure to light and air, among others[50,52].

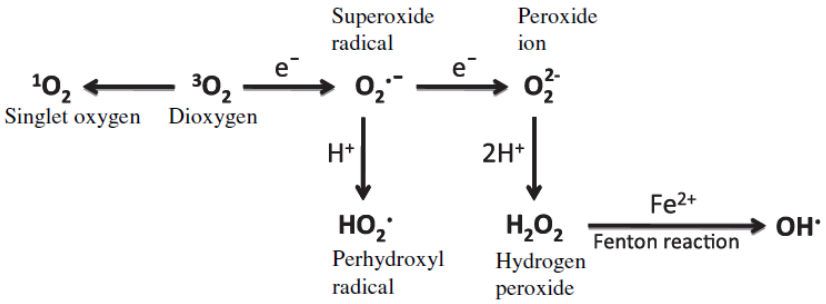


Figure 10: Generation of reactive oxygen species.
Adapted from Gill and Tuteja (2010)[68].

The average adult consumes approximately 250 grams of oxygen each day, which is reduced to metabolic water, except for 4% of it that generates superoxide (O_2^-)[66]. In healthy aerobes, the production of reactive oxygen species (ROS) is approximately balanced with the antioxidant defense systems, because it is energetically favorable to repair or replace damaged biomolecules rather than

eliminate them[65], as there are some ROS too reactive to scavenge, as in the case of the hydroxyl radical (·OH)[64].

Oxidative stress occurs when there is a serious imbalance between the reactive species production and the antioxidant defenses[64]. Alternatively, an imbalance between oxidants and antioxidants in favor of the oxidants, with potential to cellular damage[67]. Oxidants are a product of aerobic metabolism and can exist at elevated levels under pathophysiological conditions[67]. Cells responses to the presence of reactive species are very wide, which include increased proliferation, prevention of cell division, senescence, necrosis, apoptosis, or cell death mechanisms with traits of both[69]. The human diet contains several compounds of oxidant and antioxidant character[67]. This means that there are dietary compounds which may act as potential oxidants, for instance, various quinones, and substrates for enzyme systems which generate oxidants[67].

A line of defense against reactive oxygen species is to prevent their formation by for example binding of metal ions, particularly iron and copper ions[67]. Sies (1997)[67] states that metal chelation is a key step to control lipid peroxidation and DNA fragmentation. The intestinal mucosal cells are exposed to a variety of reactive intermediates and xenobiotics, and so exhibit a significant accumulation of products from oxidative damage[67]. The most efficient intercepting antioxidants are able to firstly react with initial free radicals, and secondly, interact with water-soluble compounds for their own regeneration[67]. Such intercepting chain-breaking antioxidants are often phenolic compounds[67], which are present in significant amounts in wheat and wheat-based products.

1.5. Bioavailability

Bioavailability is defined as the absorbed fraction that can be used metabolically for physiological functions[70]. The bioavailability of a nutrient depends on its bioaccessibility from the food matrix, the absorption by intestinal cells, and the transport to the body cells, among others[25]. Nutrients can be bioaccessible by some physiological processes, explicitly mastication, and in the presence of acid and digestive enzymes[25]. Food processing methods, like cooking, induce the bioaccessibility of these molecules; this is particularly seen for plant foods[25].

Antioxidants in fruits and vegetables, such as phenolic acids, are easily absorbed when weakly bound to the cell walls[71], while the absorption of lipid-soluble antioxidants (e.g. carotenoids) only occurs when they are released from the food matrix by mastication or digestion, or if solubilized in bile salt micelles[25]. In the small intestine, fibers can be in the form of soluble polymer chains, insoluble assemblies, or as a sponge-like network[25]. These structures can trap antioxidant molecules in the upper digestive tract, consequently reducing the absorption rate of antioxidants[71]. Food structure and matrix design, together with the molecular interactions, are fundamental to study the rates of nutrient digestion and bioavailability[25].

1.6. Impact of genetic and environmental factors on wheat

Abiotic stress cause large losses to agricultural production worldwide[72]. In USA, during August 2000, the combination of drought and heat stresses caused damage on agriculture costing more than $4.2 billion[73]. Drought is limiting the food production worldwide, even in the most productive regions[74]. World population is increasing and water resources for agriculture are becoming scarce, which makes the development of drought-tolerant, water-use-efficient cultivars a need[74].

In the field, a number of abiotic stresses exist simultaneously: drought (low water availability) and high temperature (or extreme temperatures) are key stress factors with high impact on crop yield[74,75]. Final yield is dependent on the sensitivity of the grain filling process to the environmental conditions[76]. Plants subjected to combined heat and drought stress presented simultaneous enhancement of respiration and suppression of photosynthesis[75]. In addition, increased sucrose levels were explained by the occurrence of the degradation of starch[75].

A study on Chilean durum wheat showed that genotype mostly defined pasta color, while the environment influenced semolina brownness and grain yield[77]. In the same study, grain yield was positively associated to the average maximum temperatures of 21-23 ºC and 300-400 mm of precipitation[77].

A review of Ron Mittler (2006) indicates that the enhancement of plants tolerance should be done towards the combination of different abiotic stresses and

not abiotic stresses alone[73]. This is because each abiotic stress condition provokes a unique response for the adaptation of the plant, which is different from the response the plant has when two or more abiotic stresses occur[73]. When different abiotic stresses co-exist, activation of different response pathways might arise which may produce synergistic or antagonistic effects on each other[73]. For example, heat stress enhances transpiration which combined with salinity or heavy metal stress could result into enhanced uptake of salts or heavy metals[73].

Abiotic stresses stimulate the production of ROS in plants that ultimately result in oxidative stress, extra- or intra-cellular, by their toxic and highly reactive action[68]. Cells have antioxidant mechanisms to defend against the ROS effects, which can be non-enzymatic (e.g. polyphenols, α-tocopherols, flavonoids, carotenoids, glutathione) or enzymatic[68].

1.7. Objective of the study

The aim of this study is to characterize four varieties of durum wheat by the quantification of phenolic acids, resistant and non-resistant starch. The characterization of the varieties Etiopia ELS-6404-115-2, Etiopia MP 3, Trinakria and Mutante 364 Hg, grown in the South of Italy, aims also to identify their potential to make part of breeding programs.

To evaluate the effects of the climatic conditions of plant growth (winter and spring of 2013 and 2014), coupled with the interaction of genotype, on the production of the healthy compounds.

2. Materials and Methods

2.1. Durum wheat samples

The whole grains of durum wheat species (*T. durum* Desf.) used in this study were kindly provided by the research institute C.R.E.A. – Cereal Research Center (C.E.R.) in Foggia (Italy), which were grown and managed under the responsibility of the Doctor Agata Rascio. Four genotypes of wheat cultivars were characterized: **Etiopia ELS**-6404-115-2 (USDA-ARS Id. type CItr 14767), **Etiopia MP 3** (USDA-ARS Id. type CItr 17240), **Mutante 364 Hg**, and **Trinakria**, an old durum wheat variety (Table 3). These varieties are under study to potentially make part of breeding programs. The wild type of Mutante 364 Hg is Trinakria, which was modified for the expression of pectin methyl esterase, and it is high in carotenoid content. The two Etiopia lines are pigmented.

Table 3**:** Variety names and number of replicates for each year and season.

Variety nr.	Variety name	2013		2014	
		Spring	Winter	Spring	Winter
1	Etiopia ELS	n=1	n=1	n=3	n=3
2	Etiopia MP 3	n=1	n=1	n=3	n=3
3	Mutante	n=1	n=1	n=3	n=3
4	Trinakria	n=1	n=1	n=3	n=3

n, number of samples.

2.2. Location and conditions of growth

The experiment was conducted in the fields of the CER, outside of Foggia (Italy), in a split-plot design with 3 repetitions, for the year 2014. Two sowing dates were the main plot: winter sowing (December 2013) and spring sowing (March 2014). In the year 2014, the main plot was divided into 3 sub-plots (1.35 x 7.50 m^2) to accommodate the wheat genotypes, while in 2013 there was a single main plot (Table 3).

Fertilization was administered at stem elongation, using 300 kg/ha ammonium nitrate. No disease infections were observed during the plant growth. In June (spring sowing) three supplemental irrigations were made in order to maintain

adequate water availability in relation to winter sowing. Maximum and minimum temperatures and rainfalls during the wheat growing period were registered every 10 days, in 2013 and 2014 (data provided by the responsible of the climatic station of CREA-CER, Dr. Antonio Troccoli). After harvesting, the seeds were conserved at 4-5 ºC.

2.3. Sample preparation

All the supplied varieties of whole wheat grain were entirely grinded using a grinding mill (Culatti Micro Hammer Mill DCFH 48) of a 0.5 mm sieve. The flour samples were stored at 10 ± 1°C.

2.4. Extraction and determination of phenolic acids

2.4.1. Preparation of standards

Stock solutions of approximately 1 mg/mL were prepared by dissolving the adequate amount of each standard in EtOH 99,8% (Sigma-Aldrich Co., St. Louis, MO, USA) with 1% HCl 6N. Standard solutions were prepared from high purity (≥ 97%): protocatechuic acid (PRO), catechin (CAT), 4-hydroxybenzoic acid (4-OHB), vanillic acid (VAN), syringic acid (SYR), di-hydroxybenzoic acids (di-OHB), caffeic acid (CAF), *p*-coumaric acid (*p*-COU), ferulic acid (FER), and sinapic acid (SIN), purchased from Fluka Chemika. Di-hydroxybenzoic acids take in account the 2,5-di-hydroxybenzoic acid, 3,5-di-hydroxybenzoic acid and 2,3-di-hydroxybenzoic acid (all three from Aldrich). All stock solutions were kept at 3 ± 1°C. The working standard solutions were properly diluted (in the concentration range of 0.01-100 mg/100 mL) with a mixture of HCl 0.06N and formic acid 0.5%.

2.4.2. Extraction of free and bound phenolic acids

The method of extraction of PAs was conceived and kindly provided by Roberto Lo Scalzo, Researcher of the CREA–IAA, based on previous experiments with modifications[51]. The extraction of phenolic acids from whole wheat grains was divided in three consecutive extractions in order to obtain three different fractions:

(a) Ethanol soluble, (b) Water soluble, and (c) Sodium hydroxide soluble, corresponding, respectively, to the (a) lowest molecular weight compounds, (b) medium molecular weight compounds, (c) highest molecular weight compounds. Initial 500 mg of whole wheat flour was extracted with 10 mL ethanol 99.8% by centrifugation (Heraeus Biofuge Stratos Centrifuge, Thermo Fisher Scientific Inc.) for 20 min at 15000 rpm, 4 °C. After filtration, the supernatant was evaporated under vacuum at 45 °C, using a Rotavapor R-3000 system, and resuspended in 10 mL distilled water. In this way it was obtained the (a) ethanol (EtOH) fraction. To the remaining residue was added 10 mL distilled water, followed by centrifugation and filtration, the same way as for ethanol extraction, obtaining (b) water (H_2O) fraction. To the third remaining pellet was added 10 mL sodium hydroxide 0.5 N, proceeded by centrifugation and filtration as for the previous extracts. The supernatant correspond to (c) sodium hydroxide (NaOH) fraction. Finally, three fractions were obtained from three consecutive extractions: EtOH, H_2O and NaOH, which were afterwards acidified with HCl 6N to get a pH of approximately 1 to maintain the stability of the phenolic acids.

2.4.3. Alkaline hydrolysis for the determination of bound phenolic acids

The three fractions obtained from the extraction step were subjected to alkaline hydrolysis. First, hydrolysis of 3 mL of each fraction with 1 mL of NaOH 6N, in the presence of 0.2 mL ascorbic acid 5% to prevent the degradation of phenolic acids. Then, the hydrolyzed fractions were heat treated in an oven for 2 hours at 85°C, after which they were acidified with 1.5 mL HCl 6N to reach a pH of approximately 1.

2.4.4. Solid-phase extraction procedure

After alkaline hydrolysis, the fractions ethanol bound (EtOH bound), water bound (H_2O bound), and sodium hydroxide bound (NaOH bound), were applied to a Solid-Phase Extraction (SPE) C_{18} cartridge in order to concentrate phenolic acids contained. The same procedure was executed for the ethanol fraction of the free phenolic acids (EtOH free) which did not suffer alkaline hydrolysis, meaning

that raw EtOH extract was used to determine free phenols. The SPE cartridge contained an octadecylsilyl C_{18} column of 50µ average particle size, 60Å average pore size, and 493 m^2/g silica surface area (Alltech Associates Inc., Deerfield, Illinois). The flow rate employed was approximately 1.50 mL/min.

The general extraction method of a reversed phase consists of four main steps: A) cartridge conditioning, B) sample application, C) wash, and D) elution, detailed in Figure 11. Water removes sugars and other polar constituents. Washing solvents should take away weakly retained interferences but should not be strong enough to elute the analyte, whereas elution solvents should be strong enough to completely elute an analyte in a small volume[78].

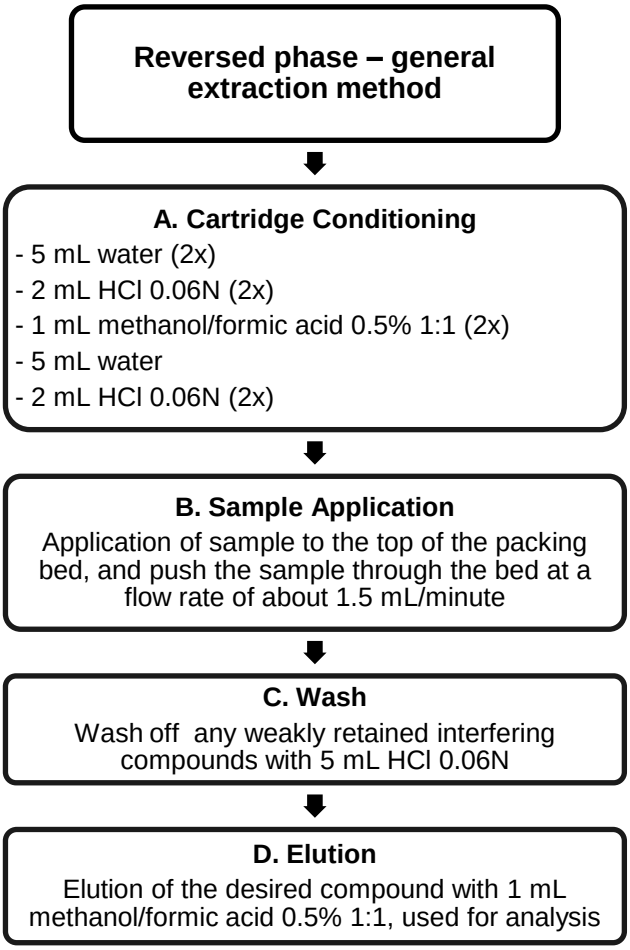


Figure 11: Procedure of the reversed phase extraction.

2.4.5. Analytical determination of phenolic acids

A High Performance Liquid Chromatography (HPLC) was carried out using a JASCO system, equipped with a quaternary gradient pump (PU-980 JASCO), a membrane degasser, a thermostated column compartment, and a diode array detection (DAD) system. The chromatographic separation of phenolic acids was carried out on a reversed-phase Inertsil® ODS-3 column (4.0 x 250 mm column size, 5 μm particle size) thermostated at 45 °C. The injection volume was 50 μL under a mobile phase gradient consisting of (A) formic acid 0.5% in bi-distilled water and (B) formic acid 0.5% in methanol at a linear flow rate of 0.7 mL/min. The gradient was as follow (A/B): 95/5 0-3 min, from 95/5 to 55/45 in 15 min, 55/45 for 17 min, from 55/45 to 95/5 in 10min, 95/5 for 10min. The identification of individual PAs was made by comparison of retention times with those of the respective standards, in addition to the spectral properties from DAD. Detection and quantification was performed at 280 nm for hydroxybenzoic acid derivatives, and 325 nm for hydroxycinnamic acid derivatives. Quantitative analysis was based on the peak area of analyte versus the peak area of respective standard. Chromatograms were recorded and evaluated by using the ChromNAV software, version 1.14.01 (spectra manager, 1.54.03; spectra analysis 1.53.05).

2.5. Determination of resistant and non-resistant starch

For the determination of resistant starch, meaning the starch that is not digested in the small intestine by monogastric animals, the assay procedure described in Megazyme resistant-starch kits (Megazyme International Ireland Ltd., Cat. No. K-RSTAR, AOAC Method 2002.02, AACC Method 32-40.01) was followed with some modifications.

2.5.1. Enzyme solutions and reagents

Porcine Pancreatic α-Amylase 164 U/mg (Megazyme) was suspended in 0.1 M, pH 6.0 sodium maleate buffer, in order to have 1g/100mL final concentration. Amyloglucosidase (AMG; EC 3.2.1.3) from *Aspergillus niger*, 59.9 U/mg, (Fluka Chemika) was suspended in 0.1M, pH 6.0 sodium maleate buffer, to get 300 U/mL

AMG. The enzyme mix α-Amylase – AMG was made by adding 1 mL AMG 300 U/mL to 1g/100mL initial pancreatic α-Amylase suspension. The enzyme mix α-Amylase – AMG was prepared immediately before use. The solution of AMG was prepared once and stored frozen between uses.

The required buffers were prepared according to the procedure described by Megazyme resistant-starch kit (Megazyme International Ireland Ltd., Cat. No. K-RSTAR, AOAC Method 2002.02, AACC Method 32-40.01) and kept at 4°C between uses.

2.5.2. Assay procedure

- **Hydrolysis and solubilization of non-resistant starch**

Approximately 100 mg ± 5 mg of wheat flour (for each variety) was weighed into a capped centrifuge tube. Each sample was dispersed in 4 mL of the enzyme mix α-Amylase – AMG, blended in a vortex mixer and placed in a 37 ºC water bath for 16h, with continuous stirring. After this, the samples were treated with 4.0 mL ethanol (99% v/v) and vigorously blended on a vortex mixer. The tubes underwent centrifugation at 1500 g for 10 min and the resulting supernatants were carefully decanted and reserved apart. The pellets were re-suspended in 2 mL 50% ethanol by vortex mixing; a further 6 mL 50% ethanol was added, followed by centrifugation at 1500 g for 10 min. The supernatants were cautiously decanted and the pellets re-suspended and centrifuged a second time, as described in the last step. The supernatants were decanted and reserved apart together with the others obtained previously.

- **Determination of resistant starch**

The pellets were re-suspended in 2 mL of 2 M KOH by stirring for 20 min in a water bath over a magnetic stirrer. After this time, 8 mL of 1.2 M pH 1.3 sodium acetate buffer and 1 mL of AMG 300 U/mL were added. The test tubes were placed in a water bath at 50 ºC for 30 min, with intermittent mixing on a vortex mixer, followed by centrifugation at 1500 g for 10 min. An aliquot of 0.9 mL of each supernatant was transferred into glass test vials in 0.1 mL of H_2SO_4 50 mN for HPLC analysis.

- **Determination of non-resistant starch**

From the combination of the supernatants obtained from the first step of this procedure, 1.2 mL were taken and adjusted to 5 mL with 100 mM pH 4.5 sodium acetate buffer in a volumetric flask. This solution was incubated with 1 mL AMG 300 U/mL at 50 ºC for 20 min. An aliquot of 0.9 mL of the incubated solution was transferred into glass test vials in 0.1 mL of H_2SO_4 50 mN for HPLC analysis.

2.5.3. Analytical determination of resistant starch

The chromatographic quantification of resistant and non-resistant starch was made in terms of glucose hydrolyzed monomers (D-glucose). Chromatography was carried out on an HPLC system equipped with a gradient pump, a thermostated column compartment, and a RI (Refractive Index) detector system. The separation was carried out on an ionic-coupled phase using two columns connected in series: Repro-Gel H^+ (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) 8.0 x 300 mm iD, 9 µm, and Polyspher® OA KC (Merck, Darmstadt, Germany) 7.8 x 300 mm iD, 9 µm, thermostated at 35 °C. The injection volume was 50 µL and the run at a linear flow rate of 0.5 mL/min, using 5mN H_2SO_4 as mobile phase. The glucose identification was made by comparison of retention times with a standard solution. Quantitative analysis was based on the peak area of analyte versus the peak area of glucose standard. To convert free D-glucose, as determined, to anhydro-D-glucose as occurs in starch we used the factor 0.9. Chromatograms were recorded and evaluated by using the Clarity Chromatography Station software (DataApex, Prague, Czech Republic).

2.6. Statistical analysis

The statistical analysis was executed by the program STATGRAPHICS Plus 5.1 (StatPoint Technologies, Inc., Warrenton, VA), and the results were reported as mean of three values ± standard deviation (SD).

To determine the effects of the genotype and the environment on the production/concentration of the total phenolic acids of each fraction and on the

content of resistant and non-resistant (soluble) starch, a three-way analysis of variance (ANOVA) was performed. The mean values associated with the main factors (variety, year, time of sowing, and the interactions of variety *x* year, variety *x* sowing, year *x* sowing, and variety *x* year *x* sowing), were evaluated using the Fisher's least significant difference (LSD) method and statistically significant differences were accepted at the minimum probability level of $p<0.05$. Additionally, for each variety a two-way ANOVA was performed on each measured phenolic acid to test the effect of the year and time of sowing and their interaction.

Each fraction of the total phenolic acids and the resistant and non-resistant starch content were subjected to discriminant analysis to verify which compound or groups of compounds best distinguished among the groups examined. Canonical discriminant analysis was used for the graphical representation of the groups' distribution by plotting the individual scores for the two principal functions obtained from the model (software PAST version 2.16).

3. Results

The results that follow show the identification and quantification of free and bound phenolic acids (PAs) in four cultivars of durum wheat, extracted in serie by ethanol, water and sodium hydroxide. By doing this, we can estimate the content and character of the phenolic acids bounded to low, medium and high molecular weight molecules, respectively. Besides bounded PAs we also analyzed the ethanol extract for free phenolic acids. Resistant and non-resistant starch content was determined from the same four cultivars of durum wheat.

Our aim was not only to analyze the content of healthy compounds abovementioned, but also to understand how environment affects their presence in the cultivars of durum wheat.

3.1. Environmental conditions

Trends of temperature and rainfalls during the growing period of wheat were registered and are shown in Figure 12 and Figure 13, respectively, for 2013 and 2014.

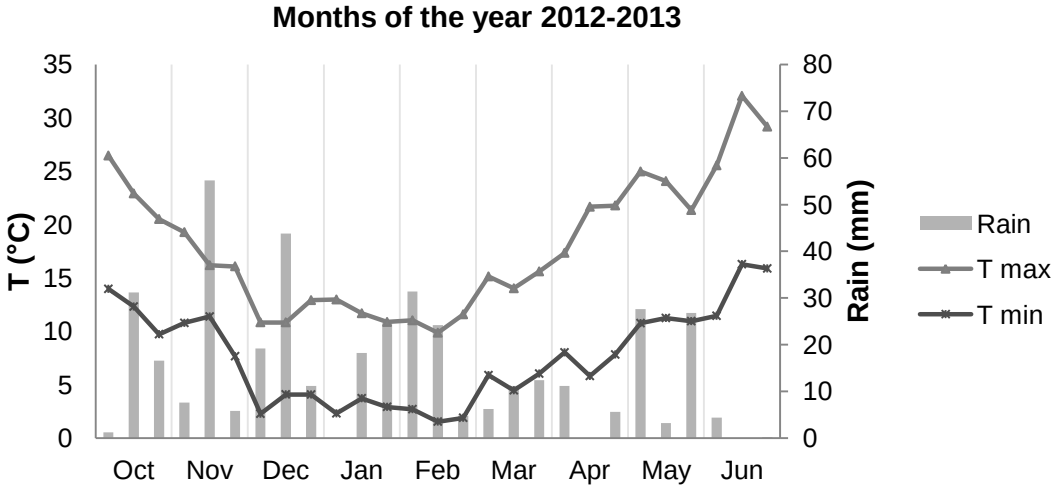


Figure 12: Trends of temperature and rainfalls during wheat growing preriod in 2013, based on records made every 10 days (data provided by Dr. Antonio Troccoli, responsible of the climatic station of CREA-CER).

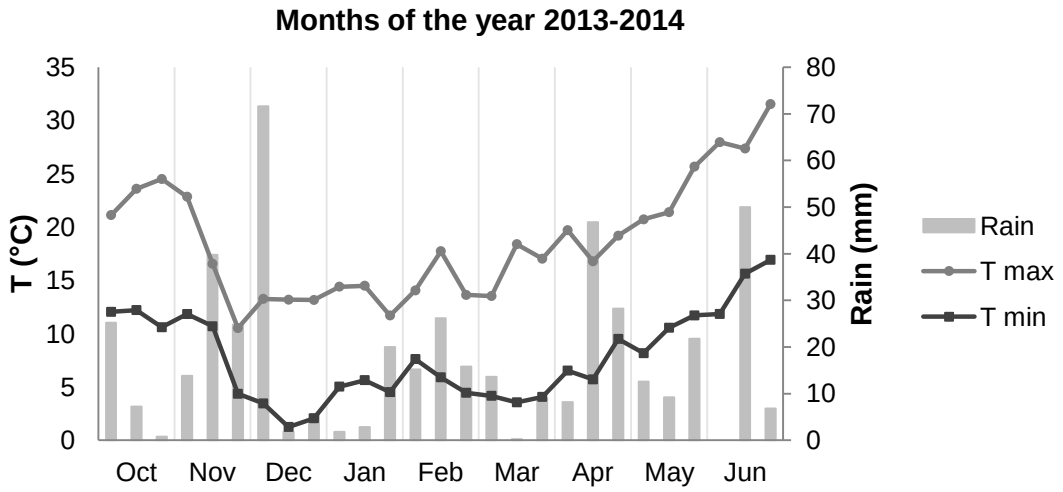


Figure 13: Trends of temperature and rainfalls during wheat growing period in 2014, based on records made every 10 days (data provided by Dr. Antonio Troccoli, responsible of the climatic station of CREA-CER).

Table 4: Mean temperatures detected every month of each year, 2013 and 2014.

Month	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Mean T (ºC) 2013	17,65	13,57	7,50	7,42	6,44	10,20	13,74	17,23	21,73
Mean T (ºC) 2014	11,62	8,96	2,25	5,06	5,99	3,93	7,25	10,14	14,80

The grain filling period of the winter sown wheat was in May and of spring sown wheat was in June. In May, rainfalls of 2013 and 2014 were quite similar, on the contrary to June which was about 10 times more rainy in 2014 (Figure 12 and Figure 13). Mean temperatures of May and June decreased from 2013 to 2014 of approximately 7 ºC (Table 4). In general, 2013 was warmer than 2014. The rainfalls tendency was comparable in both years, except in April, which was rainier in 2014.

3.2. Determination of phenolic acids

The determination of bound PAs requires an hydrolysis in order to release esterified PAs from the polymeric constituents of the cell-wall. The alkaline hydrolysis, under our procedure, provided better definition of peaks in HPLC-DAD than acid hydrolysis did, and for this reason we present the results relative to the alkaline hydrolysis.

Chromatograms of the specific standards of phenolic acids are shown in Figure 14 and Figure 15. Figure 16 shows a typical chromatogram obtained from EtOH fraction before alkaline hydrolysis, representing free PAs. Figure 17, Figure 18 and Figure 19 represent typical chromatograms of the hydrolysed samples of durum wheat, representing respectively the EtOH, H_2O and NaOH soluble bound PAs.

The main figures have the same numerical scale, while upper right figures represent a zoom of the peaks' area of interest (in lowercase letters). The absorbance was taken at 280 nm for the hydroxybenzoic acids and 325 nm for the hydroxycinnamic acids.

The chromatograms are given by the cultivar Etiopia MP 3 of spring 2014. The main peaks found were ferulic (FER), di-hydroxybenzoic acids (di-OHB), sinapic (SIN), followed by catechin (CAT). FER was the most abundant phenolic acid in the NaOH fraction.

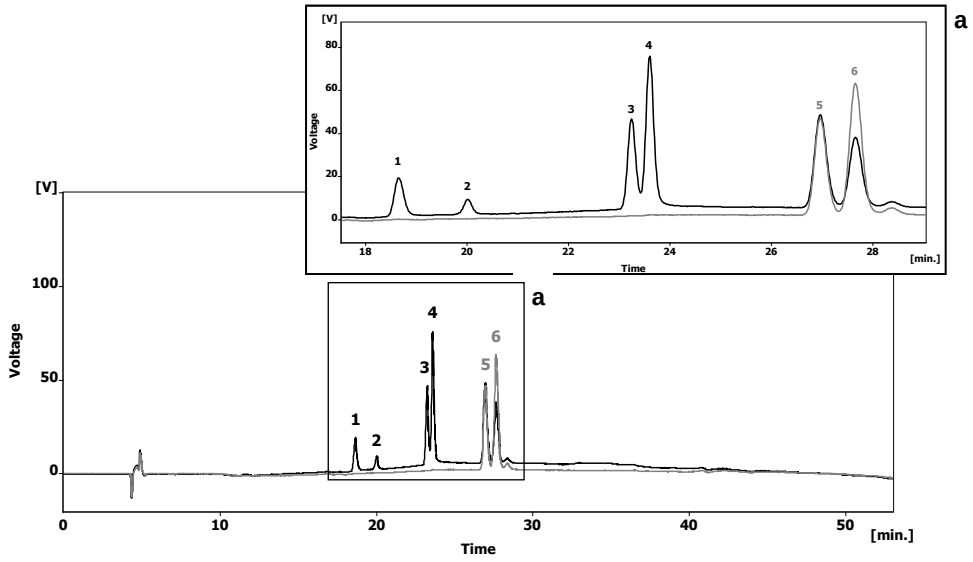


Figure 14: Standards of phenolic acids. 1- Protocatechuic acid, 2- Catechin, 3- Vanillic acid, 4- Syringic acid, 5- p-Coumaric acid, 6- Ferulic acid. Absorbance at 280 nm (black line) and 325 nm (grey line).

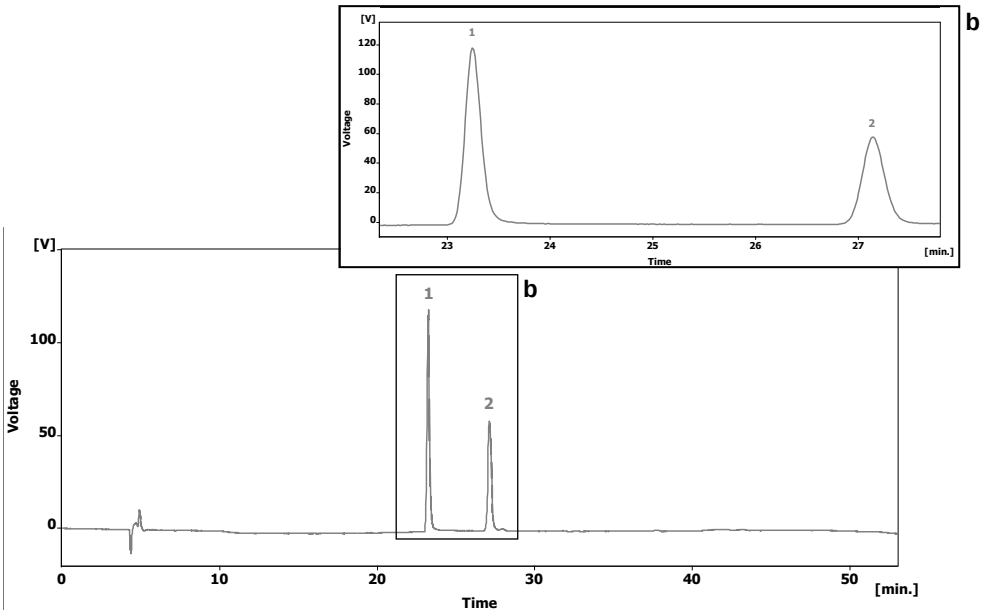


Figure 15: Standards of phenolic acids. 1- Caffeic acid, 2- Sinapic acid. Absorbance at 325 nm (grey line).

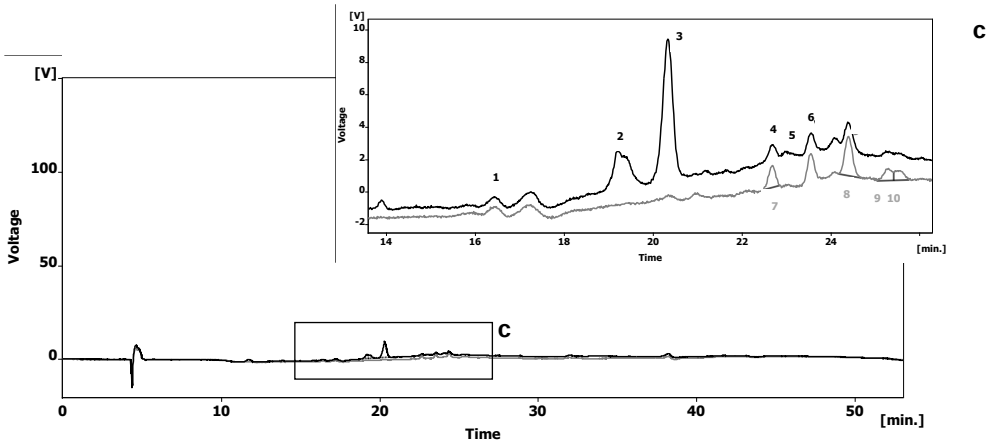


Figure 16: Chromatogram of a durum wheat sample, representing the free phenolic acids (EtOH fraction). 1-Protocatechuic acid, 2- Catechin, 3- 4-Hydroxybenzoic acid, 4-Vanillic acid, 5- Syringic acid, 6- Di-hydroxybenzoic acid, 7- Caffeic acid, 8- p-Coumaric acid, 9-Sinapic acid, 10- Ferulic acid. Absorbance at 280 nm (black line) and 325 nm (grey line).

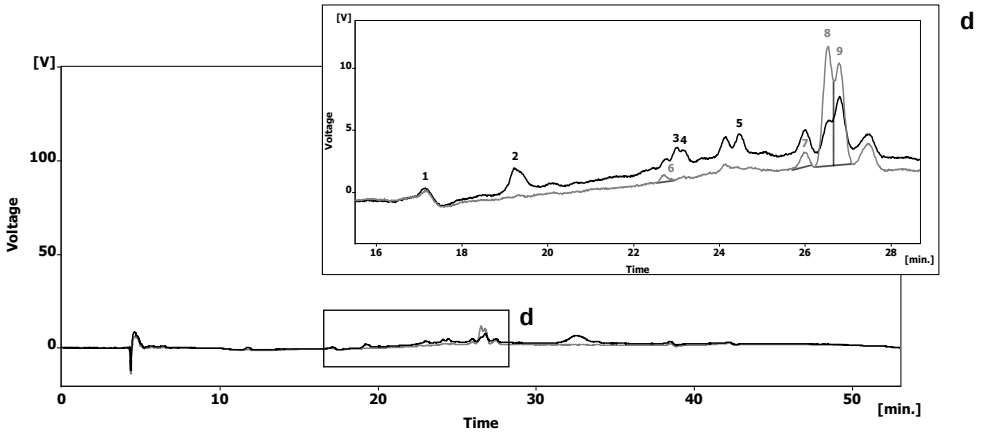


Figure 17: Chromatogram of a durum wheat sample, representing the bound phenolic acids soluble in EtOH. 1- Protocatechuic acid, 2- Catechin, 3- Vanillic acid, 4- Syringic acid, 5- Di-hydroxybenzoic acid, 6- Caffeic acid, 7- p-Coumaric acid, 8- Sinapic acid, 9- Ferulic acid. Absorbance at 280 nm (black line) and 325 nm (grey line).

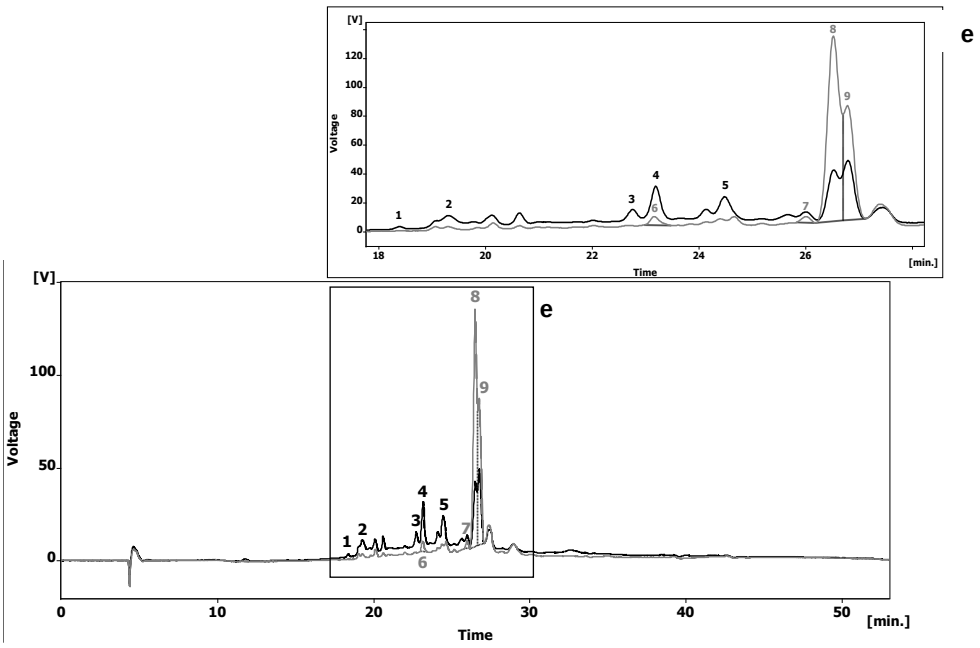


Figure 18: Chromatogram of a durum wheat sample, representing the bound phenolic acids soluble in H_2O. 1- Protocatechuic acid, 2- Catechin, 3- Vanillic acid, 4- Syringic acid, 5- Di-hydroxybenzoic acid, 6- Caffeic acid, 7- p-Coumaric acid, 8- Sinapic acid, 9- Ferulic acid. Absorbance at 280 nm (black line) and 325 nm (grey line).

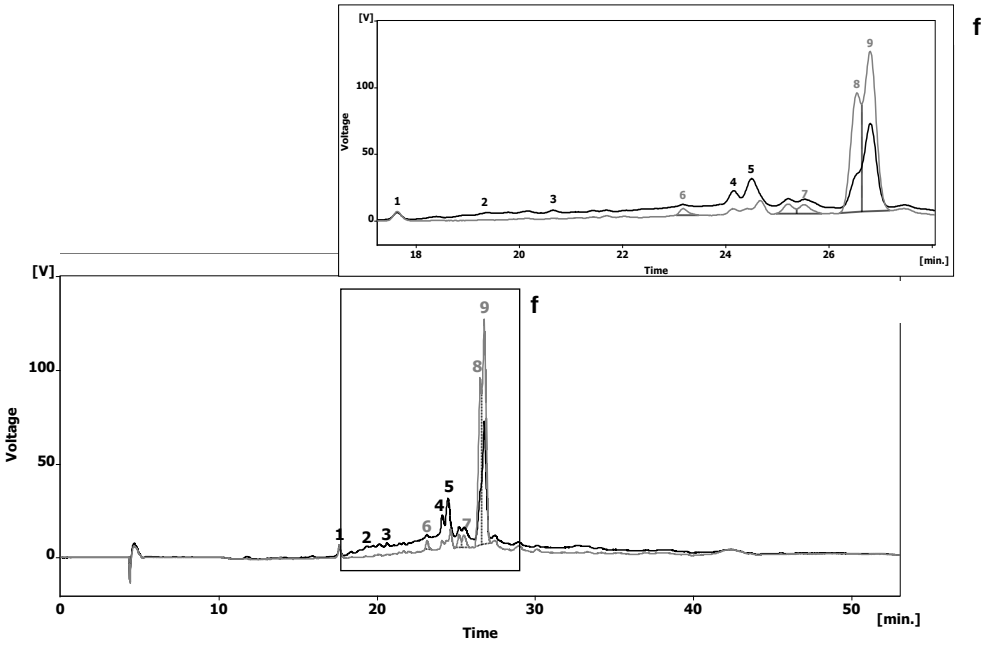


Figure 19: Chromatogram of a durum wheat sample, representing the bound phenolic acids soluble in NaOH. 1-Protocatechuic acid, 2- Catechin, 3- 4-hydroxybenzoic acid, 4-Vanillic acid, 5- Syringic acid, 6- Caffeic acid, 7- p-Coumaric acid, 8- Sinapic acid, 9-Ferulic acid. Absorbance at 280 nm (black line) and 325 nm (grey line).

A profile was observed in the H_2O fraction, in which the peak height of sinapic acid was higher than the one of ferulic acid (peaks 8 and 9, Figure 18). Reversely to the profile of the NaOH fraction, in which the peak height of ferulic acid was higher than that of sinapic acid (peaks 8 and 9, Figure 19).

3.2.1. Free phenolic acids

Identification and quantification of free phenolic acids are evidenced in Table 5 (see mean ± SD in Table 12, in Appendix). The major free phenolic acids were CAT, di-OHB, 4-OHB and FER. The concentration of free PAs ranged from 0.019 µg/g SIN, in Trinakria, to12.102 µg/g di-OHB, in Etiopia ELS.

For all the varieties sown in both years and seasons, the difference between the content of total phenolic acids and FER is very high, meaning that the presence of other and diverse phenolic acids is accentuated (Figure 20). Moreover, FER was more visible for Mutante in spring and for Trinakria in 2014 (Figure 20).

Total free phenolic acids were calculated as the sum of all detected PAs for each variety sown in winter and spring, in the years 2013 and 2014. The highest value was observed for Etiopia MP 3 in both seasons of 2014 and for Etiopia ELS (Figure 21).

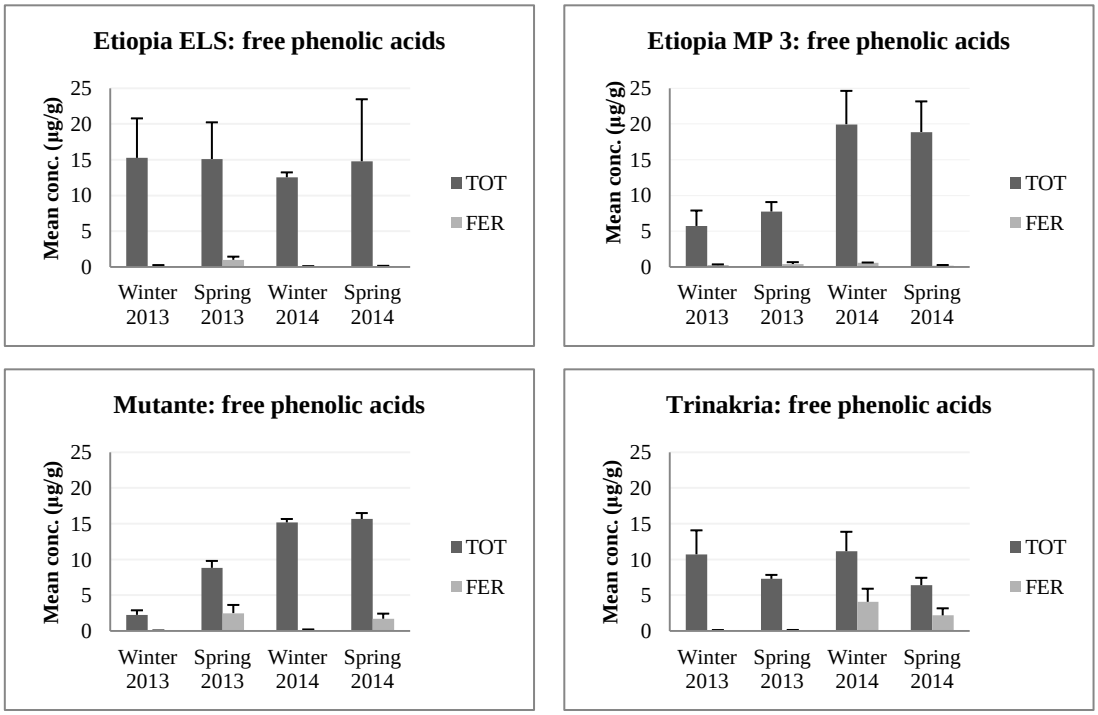


Figure 20 : Mean concentration of the total (TOT) free phenolic acids alongside to the mean concentration of ferulic acid (FER) found in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

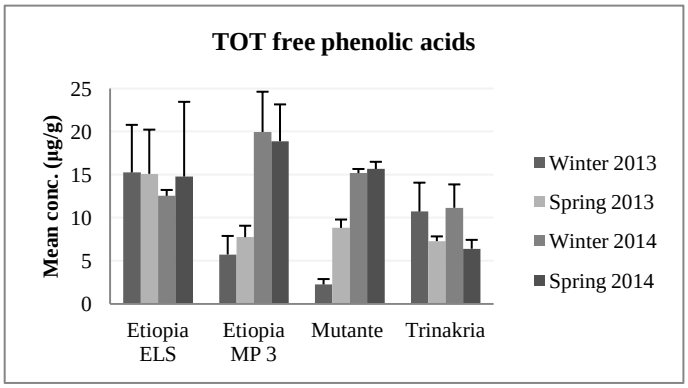


Figure 21: Total (TOT) free phenolic acids present in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

In both seasons of 2013, the content of free phenolic acids was significantly or just lower than in 2014 for all the varieties except for Etiopia ELS. In 2014, Trinakria had the lowest content of free PAs and Etiopia MP 3 the highest.

The concentration of some free PAs in the genotype Etiopia ELS were affected by the year and by the interaction of the year with the season sowing (Table 5). 4-OHB was significantly higher in year 2013 (mean 1.41 µg/g) than that of 2014 (mean 0.16) ($p<0.05$). While, spring of 2013 produced significantly more FER than the other combinations of year x sowing ($p<0.05$).

For Etiopia MP 3, the year 2014 presented significantly higher content of single and TOT PAs (Table 5). PRO concentration in this cultivar was significantly higher in spring (mean 0.33 µg/g) than in winter (mean 0.11 µg/g) ($p<0.05$). The concentrations of 4-OHB, SYR, p-COU, and SIN were significantly or just higher in spring of 2014. The same happened for the TOT free phenolic acids in spring and winter of 2014 with a concentration of, respectively, 18.87 µg/g and 19.92 µg/g, which were significantly higher than in both seasons of 2013 (spring, 7.74 µg/g; winter, 5.73 µg/g).

For Mutante, the year 2014 contained significantly higher concentrations of PRO, CAT, 4-OHB, and di-OHB than the year 2013, while spring sowing caused a significantly higher content of SIN (mean 0.26 µg/g) ($p<0.01$). For this genotype, with higher or lower extent, the interactions of year with sowing affected differently the production of PAs and a tendency was not found.

Trinakria was the only genotype in which 2013 produced significantly higher concentrations of free PAs, specifically for SYR and di-OHB (Table 5). FER however was significantly higher in 2014 ($p<0.05$). Winter provided for a significantly higher content of TOT free phenolic acids (winter mean 10.93 µg/g vs. spring mean 6.84 µg/g; $p<0.05$) and CAT, on the opposite of 4-OHB, significantly higher in spring. Winter 2014 was the most relevant interaction year x sowing for a higher production of the phenolic acids CAF and FER, while spring 2013 was for SYR and p-COU ($p<0.05$).

Table 5: Statistical analysis of the content of free phenolic acids present in four durum wheat cultivars, sown in winter and spring of 2013 and 2014.

			Mean concentration (µg/g)										
Cultivar	Year	Season	PRO	CAT	4-OHB	VAN	SYR	di-OHB	CAF	p-COU	SIN	FER	TOT
Etiopia ELS	2013	Winter	0.63	nd	1.80	0.25	0.17	12.10	0.06	nd	0.08	0.16 a	15.25
		Spring	0.33	10.32	1.43	0.01	1.17	0.78	0.05	nd	nd	0.99 b	15.09
	2014	Winter	0.19	4.35	0.21	0.18	0.19	7.20	0.07	nd	0.06	0.10 a	12.53
		Spring	0.19	6.24	0.11	0.46	0.15	7.38	0.07	nd	0.06	0.13 a	14.77
		year	ns	ns	*	ns	ns	ns	ns	-	ns	ns	ns
		sowing	ns	ns	ns	ns	ns	ns	ns	-	ns	ns	ns
		year x sowing	ns	ns	ns	ns	ns	ns	ns	-	ns	*	ns
Etiopia MP 3	2013	Winter	0.14	1.97	0.29 a	0.33	0.09 a	2.17	0.37	0.12 a	nd a	0.25	5.73 a
		Spring	0.40	2.64	0.83 a	0.64	0.14 a	2.48	0.08	nd a	0.13 b	0.40	7.74 a
	2014	Winter	0.05	10.67	3.31 b	0.58	0.24 a	4.32	0.06	nd a	0.13 b	0.57	19.92 b
		Spring	0.26	5.71	4.44 b	0.46	0.56 b	6.35	0.26	0.50 b	0.13 b	0.20	18.87 b
		year	ns	*	**	ns	*	*	ns	ns	ns	ns	**
		sowing	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
		year x sowing	ns	ns	*	ns	**	ns	ns	**	**	ns	*
Mutante	2013	Winter	0.08 a	0.39 a	0.21	0.32 a	0.12 a	1.03	0.04 a	nd a	nd a	0.05	2.25
		Spring	0.17 a	0.27 a	0.56	2.04 b	1.05 b	1.68	0.38 a	nd a	0.20 b	2.49	8.82
	2014	Winter	0.83 b	2.85 a	0.95	0.22 a	0.37 a	7.32	2.49 b	nd a	nd a	0.16	15.17
		Spring	1.05 b	5.84 b	1.17	0.27 a	0.31 a	4.10	0.74 a	0.17 b	0.32 b	1.70	15.65
		year	**	**	*	ns	ns	*	ns	ns	ns	ns	ns
		sowing	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns
		year x sowing	*	*	ns	***	**	ns	*	*	*	ns	ns
Trinakria	2013	Winter	0.07	5.11	0.12	0.43	0.29 a	4.46	0.10 a	nd a	0.04	0.10 a	10.71
		Spring	0.71	0.93	0.57	0.38	0.66 b	3.76	0.10 a	0.06 b	0.02	0.10 a	7.28
	2014	Winter	0.49	2.85	0.05	0.52	0.15 a	2.53	0.48 b	nd a	0.03	4.07 b	11.15
		Spring	0.24	0.93	0.43	0.39	0.11 a	2.01	0.10 a	nd a	nd	2.17 ab	6.39
		year	ns	ns	ns	ns	*	*	ns	ns	ns	*	ns
		sowing	ns	*	*	ns	ns	ns	ns	ns	ns	ns	*
		year x sowing	ns	ns	ns	ns	*	ns	*	*	ns	*	ns

Significance (n=3) is shown as * for p<0.05, ** for p<0.01, and *** for p<0.001, ns when means are not significantly different. nd, not detected.

3.2.2. Bound phenolic acids

3.2.2.1. EtOH fraction

Identification and quantification of phenolic acids bound to structures soluble in EtOH are evidenced in Table 6 (see mean ± SD in Table 13, in Appendix). The major bound phenolic acids detected in the EtOH fraction after alkaline hydrolysis were CAT, di-OHB and FER. The concentration of bound PAs ranged from 0.026 µg/g CAF, in Mutante, to 14.736 µg/g di-OHB, in Mutante.

After alkaline hydrolysis, the content of FER has increased when compared with free FER of the EtOH fraction before hydrolysis. The totality of phenolic acids bound to structures soluble in EtOH however have not increased if compared to the total free phenolic acids, with the exception of Trinakria sown in 2013 that presented higher content of total bound PAs than total free (Figure 21 and Figure 23). FER increase was more accentuated for Etiopia MP 3 and Trinakria in 2013 (Figure 22).

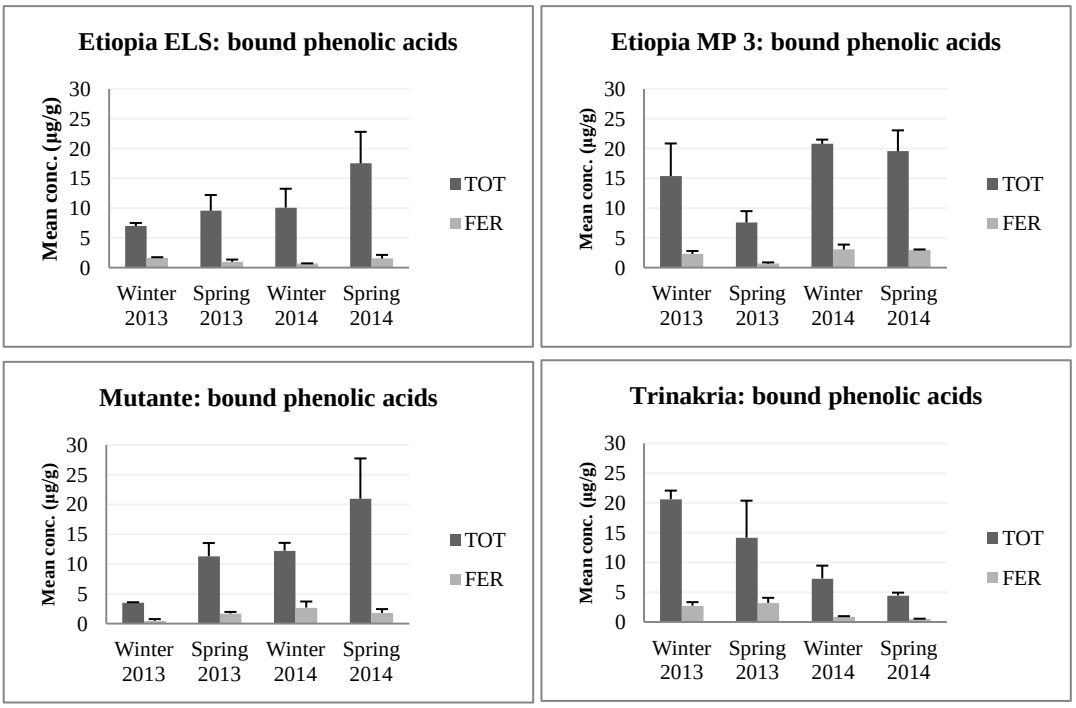


Figure 22 : Mean concentration of the total (TOT) phenolic acids bound to structures soluble in EtOH alongside to the mean concentration of ferulic acid (FER) found in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

The total bound PAs of Etiopia ELS and Mutante have increased from winter 2013 to winter 2014 and from spring 2013 to spring 2014. In Etiopia MP 3 the same tendency was observed for the spring sowing season, but not for winter. Trinakria had an opposite response to the environmental conditions and to the year and season, because winter and spring of 2013 were the most productive on total bound PAs (Figure 23). Overall, Etiopia MP 3 had the highest production of TOT bound PAs, in both seasons and years.

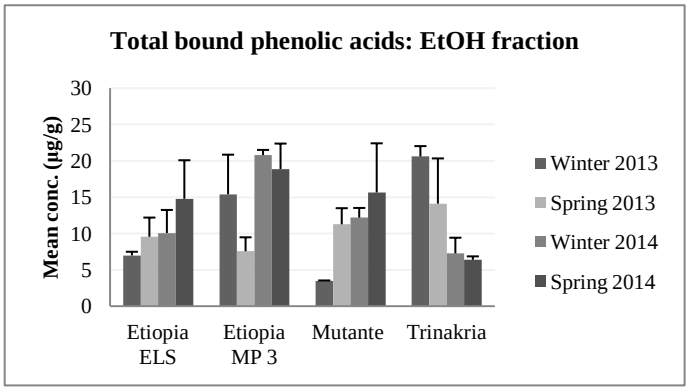


Figure 23: Mean concentration of the total phenolic acids bound to structures soluble in EtOH present in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

The genotype Etiopia ELS produced a significantly higher content of CAT in 2014 (mean 3.398 µg/g vs. 2013 mean 0.621 µg/g), while the rest was not influenced by the year (Table 6). In spring, the concentrations of PRO, 4-OHB, and SIN were significantly superior than those observed in winter ($p<0.05$). The interaction year x sowing caused significant differences for di-OHB, which content of 8.93 µg/g was significantly higher in spring 2014 ($p<0.05$).

For the genotype Etiopia MP 3, the year 2014 promoted significantly higher concentrations of CAT, SYR, FER, and the TOT bound phenolic acids (Table 6). On the contrary, CAF was significantly higher in 2013 but with a general lower concentration ($p<0.05$).The sowing season alone did not affect the content of PAs in Etiopia MP 3. FER was among the most produced phenolic acids of this cultivar in all the combinations of year and season sowing, except the spring of 2013 which has given a significantly lower content ($p<0.05$).

Mutante, in 2014, provided for significantly higher contents of CAT, 4-OHB, and TOT bound phenolic acids than in 2013 (Table 6). Spring permitted a significantly higher concentration of SIN and TOT bound phenolic acids (both $p<0.05$). The statistical results from the interaction year x sowing showed a significantly higher concentration of CAT in winter and spring of 2014 ($p<0.05$), p-COU in spring 2013 ($p<0.01$), and TOT phenolic acids in all seasons-years except for winter 2013, containing the lower concentration of PAs observed among all the varieties ($p<0.05$).

With Trinakria, 2013 was the most significantly productive year for CAT, VAN, CAF, SIN, FER and the TOT phenolic acids ($p<0.01$, except for CAT with $p<0.05$) (Table 6). The content of CAT was significantly higher in winter ($p<0.01$), on the opposite to 4-OHB that was present only in spring. From the statistical interaction year x sowing it was observed that, winter 2013 prevailed over the other groups for the content on CAT ($p<0.001$), VAN and TOT phenolic acids, while for SIN and FER, both winter and spring of 2013 were favourable.

Table 6: Statistical analysis of the content of phenolic acids bound to structures soluble in EtOH, present in four cultivars of durum wheat sown in winter and spring of 2013 and 2014.

			Mean concentration (µg/g)										
Cultivar	Year	Season	PRO	CAT	4-OHB	VAN	SYR	di-OHB	CAF	p-COU	SIN	FER	TOT
Etiopia ELS	2013	Winter	0.11	0.69	0.18	0.41	0.52	2.85 [a]	0.09	0.13	0.39	1.62	6.99
		Spring	0.35	0.55	1.15	1.14	0.29	3.70 [a]	0.45	nd	0.97	0.97	9.56
	2014	Winter	0.32	3.76	0.14	0.36	0.32	3.90 [a]	0.09	nd	0.49	0.69	10.07
		Spring	0.53	3.04	0.45	0.61	0.37	8.93 [b]	0.12	0.08	1.83	1.54	17.50
		year	ns	2014*	ns	ns	ns	ns	ns	ns	ns	ns	ns
		sowing	*	ns	*	ns	ns	ns	ns	ns	*	ns	ns
		year x sowing	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
Etiopia MP 3	2013	Winter	0.26	2.14	0.15	1.13	0.14 [a]	7.12	0.35	0.26	1.55	2.29 [b]	15.39
		Spring	0.16	2.40	0.05	0.68	0.10 [a]	2.27	0.31	0.08	0.80	0.71 [a]	7.57
	2014	Winter	0.08	9.78	0.09	0.66	0.41 [b]	4.52	0.11	0.38	1.70	3.04 [b]	20.78
		Spring	0.93	6.22	0.08	0.83	0.66 [b]	5.37	0.12	0.47	1.92	2.94 [b]	19.54
		year	ns	*	ns	ns	**	ns	*	ns	ns	*	*
		sowing	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
		year x sowing	ns	ns	ns	ns	*	ns	ns	ns	ns	*	ns
Mutante	2013	Winter	0.16	0.24 [a]	nd	0.35	0.06	2.04	0.03	0.08 [a]	0.09	0.46	3.50 [a]
		Spring	0.71	0.18 [a]	nd	0.91	0.60	6.24	0.24	0.36 [b]	0.37	1.67	11.27 [ab]
	2014	Winter	0.34	1.88 [b]	0.13	0.67	0.78	5.19	0.12	0.11 [a]	0.31	2.66	12.19 [ab]
		Spring	0.33	2.04 [b]	0.14	0.76	0.39	14.74	0.15	0.10 [a]	0.53	1.77	20.93 [b]
		year	ns	**	**	ns	ns	ns	ns	ns	ns	ns	*
		sowing	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*
		year x sowing	ns	*	ns	ns	ns	ns	ns	**	ns	ns	*
Trinakria	2013	Winter	0.22	5.83 [c]	nd	1.34 [b]	0.56	6.46	0.50	nd [a]	2.98 [b]	2.71 [b]	20.59 [c]
		Spring	0.78	1.37 [a]	0.10	0.81 [ab]	0.56	2.63	0.57	0.49 [b]	3.63 [b]	3.17 [b]	14.11 [bc]
	2014	Winter	0.51	2.71 [b]	nd	0.28 [a]	0.22	2.11	0.03	nd [a]	0.57 [a]	0.85 [a]	7.27 [ab]
		Spring	0.16	0.91 [a]	0.06	0.21 [a]	0.41	1.73	0.03	nd [a]	0.41 [a]	0.50 [a]	4.42 [a]
		year	ns	*	ns	**	ns	ns	**	ns	**	**	**
		sowing	ns	**	*	ns	ns	ns	ns	ns	ns	ns	ns
		year x sowing	ns	***	ns	*	ns	ns	ns	*	*	*	*

Significance (n=3) is shown as * for p<0.05, ** for p<0.01, and *** for p<0.001, ns when means are not significantly different. nd, not detected.

3.2.2.2. Water fraction

Identification and quantification of phenolic acids bound to structures soluble in H_2O are evidenced in Table 7 (see mean ± SD in Table 14, in Appendix). The major bound PAs detected in the H_2O fraction after alkaline hydrolysis were derivatives of di-OHB, SIN and FER. Although, concentrations of VAN and SYR generally increased in relation to the EtOH fraction. The concentration of bound PAs ranged from 0.115 µg/g p-COU, in Etiopia ELS, to 71.856 µg/g di-OHB, in Etiopia MP 3.

The content of FER in the H_2O fraction was about five times higher than in the EtOH fraction. Etiopia ELS in winter 2013 contained the highest concentration of FER (32.64±2.20 µg/g) (Figure 24).

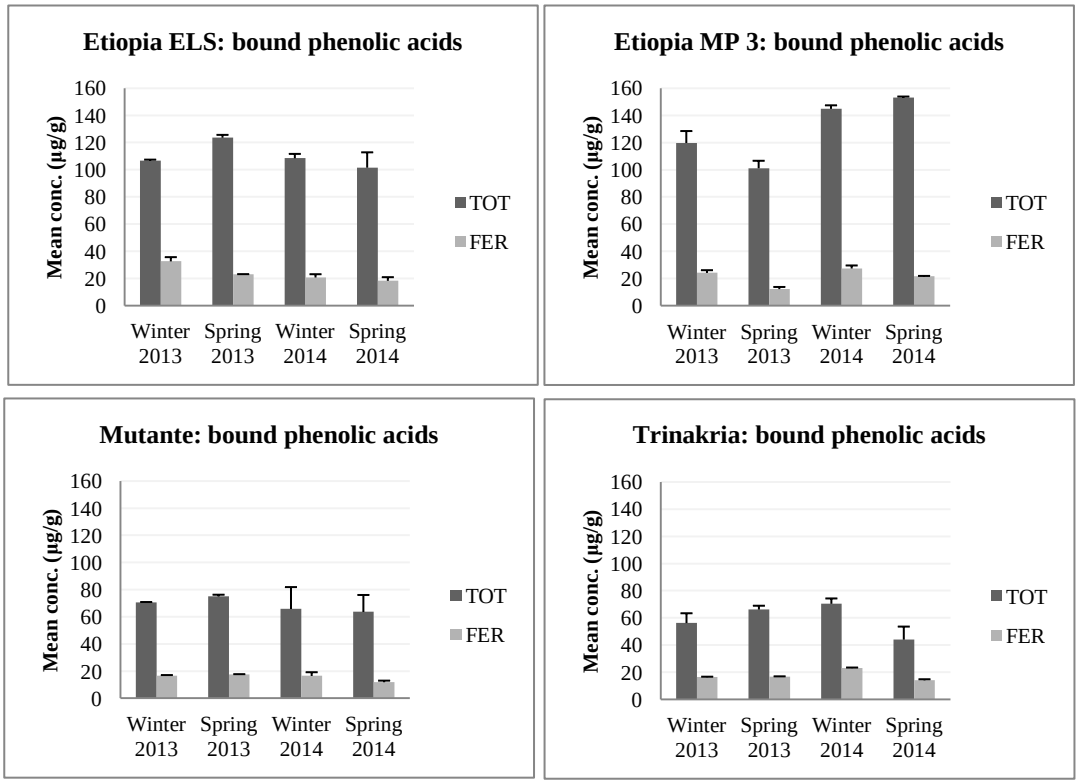


Figure 24: Mean concentration of the total (TOT) phenolic acids bound to structures soluble in H_2O alongside to the mean concentration of ferulic acid (FER) found in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

The total bound phenolic acids were 3 to 7.5 times higher than the total bound PAs obtained in the EtOH fraction. The total bound PAs of Etiopia MP 3 have

increased from winter 2013 to winter 2014 and from spring 2013 to spring 2014. The behavior of Etiopia ELS and Trinakria from spring 2013 to spring 2014 was towards a decrease on the total bound PAs. Overall, total bound PAs detected in Mutante and Trinakria were significantly lower than that of the two Ethiopian lines (Figure 25).

Etiopia MP 3 was the most highly productive genotype on total bound PAs, with 144.88 ± 1.85 µg/g in winter 2014 and 153.04 ± 0.64 µg/g in spring 2014.

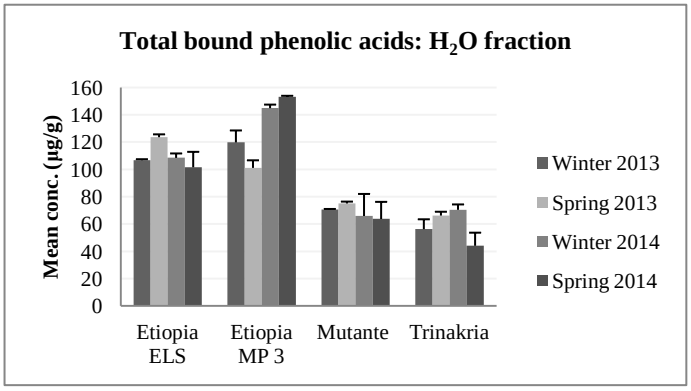


Figure 25: Mean concentration of the total phenolic acids bound to structures soluble in H_2O present in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

Etiopia ELS had significantly higher concentrations of VAN, CAF, and FER in 2013, on the contrary to CAT, significantly higher in 2014 (Table 7). The content of 4-OHB, p-COU and FER were significantly increased by the sowing in winter and CAF by spring. Statistically, the interaction winter 2014 provoked a significant increase on the concentrations of CAT and p-COU, winter 2013 for FER, and spring 2013 for VAN, di-OHB and CAF.

Etiopia MP 3 in 2014 provided for higher concentrations of CAT, VAN, SYR, di-OHB, CAF, FER and total bound phenolic acids (Table 7). In 2014, TOT bound PAs mean concentration of 148.96 µg/g was the highest observed in the H_2O fraction. FER was significantly increased by winter sowing ($p<0.01$) and by the interaction winter 2014 ($p<0.01$). The interactions 2014 spring and 2014 winter significantly elevated the content of VAN and TOT bound phenolic acids, 2014

spring for SYR, di-OHB and p-COU; CAF was significantly lower for 2013 spring relative to the other year-season sowing interactions.

For Mutante, the concentration of SIN was significantly higher in 2013 and by the statistical interaction of winter 2013 ($p<0.001$) (Table 7). CAT was significantly increased by winter sowing. PRO however was significantly higher in spring 2014 ($p<0.01$).

Trinakria, in 2014, provided for significantly higher contents of PRO and CAF, while in 2013 for significantly more SIN (Table 7). Spring season contributed to a significant increase on SYR concentration. CAF was significantly higher by the interaction 2014 spring and 2014 winter, p-COU by 2013 spring, SIN by 2013 spring and 2013 winter and FER by 2014 winter.

In the H_2O fraction, SIN and FER with around 20 µg/g each were the most important phenolic acids detected in all the varieties, besides a similar amount of di-OHB.

Table 7: Statistical analysis of the content of phenolic acids bound to structures soluble in H_2O, present in four cultivars of durum wheat sown in winter and spring of 2013 and 2014.

			Mean concentration (µg/g)										
Cultivar	year	season	PRO	CAT	4-OHB	VAN	SYR	di-OHB	CAF	p-COU	SIN	FER	TOT
Etiopia ELS	2013	Winter	0.31	2.72 a	1.66	2.98 a	4.21	36.24 a	1.26 bc	0.12 ab	24.65	32.64 b	106.78
		Spring	1.33	2.81 a	1.15	5.41 b	6.45	57.47 c	1.64 c	nd a	24.39	22.98 a	123.63
	2014	Winter	0.69	9.90 b	1.67	2.33 a	3.76	50.80 bc	0.62 a	0.20 b	17.74	20.76 a	108.48
		Spring	0.46	3.74 a	1.08	1.93 a	3.00	48.41 b	0.98 ab	nd a	23.64	18.34 a	101.57
		year	ns	*	ns	*	ns	ns	**	ns	ns	*	ns
		sowing	ns	ns	*	ns	ns	ns	*	**	ns	*	ns
		year x sowing	ns	**	ns	*	ns	**	*	*	ns	*	ns
Etiopia MP 3	2013	Winter	0.14	4.04	3.01	3.09 a	5.01 b	54.51 a	1.24 b	1.16 ab	23.36	24.22 bc	119.77 b
		Spring	0.74	1.66	1.23	2.85 a	2.98 a	57.49 ab	0.43 a	0.84 a	20.59	12.34 a	101.16 a
	2014	Winter	0.72	12.92	2.28	5.79 b	6.13 c	65.25 bc	1.92 b	1.10 a	21.30	27.47 c	144.88 c
		Spring	0.76	13.33	2.72	6.28 b	7.76 d	71.86 c	1.86 b	1.51 b	25.20	21.77 b	153.04 c
		year	ns	*	ns	**	*	**	**	ns	ns	*	**
		sowing	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns
		year x sowing	ns	ns	ns	*	***	*	*	*	ns	**	**
Mutante	2013	Winter	0.41 a	5.09	0.87	1.67	1.80	30.34	0.44	0.65	12.67 c	16.68	70.63
		Spring	0.46 a	2.30	1.01	3.92	3.09	33.49	1.11	0.77	11.45 b	17.52	75.11
	2014	Winter	0.15 a	5.70	1.80	1.69	2.35	26.47	0.95	1.27	8.91 a	16.64	65.93
		Spring	2.57 b	3.01	1.33	1.28	1.97	31.20	0.82	0.52	9.02 a	12.01	63.73
		year	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns
		sowing	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
		year x sowing	**	ns	ns	ns	ns	ns	ns	ns	***	ns	ns
Trinakria	2013	Winter	0.14	3.65	0.66	1.38	2.63	15.81	0.29 a	nd a	15.16 c	16.56 b	56.28
		Spring	0.14	3.67	0.85	1.55	3.58	22.67	0.42 a	0.75 b	15.91 c	16.68 b	66.23
	2014	Winter	1.06	5.12	1.18	2.30	2.33	24.00	1.14 b	nd a	10.15 b	23.20 c	70.46
		Spring	0.47	2.90	1.08	1.43	3.07	13.47	0.94 b	0.13 a	6.38 a	14.24 a	44.12
		year	*	ns	ns	ns	ns	ns	***	ns	**	ns	ns
		sowing	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
		year x sowing	ns	ns	ns	ns	ns	ns	**	*	***	***	ns

Significance (n=3) is shown as * for p<0.05, ** for p<0.01, and *** for p<0.001, ns when means are not significantly different. nd, not detected.

3.2.2.3. NaOH fraction

Identification and quantification of phenolic acids bound to structures soluble in NaOH are evidenced in Table 8 (see mean ± SD in Table 15, in Appendix). The major bound PAs detected in the NaOH fraction after alkaline hydrolysis were the derivatives of di-OHB, SIN and FER. The concentration of bound PAs ranged from 0.153 µg/g 4-OHB, in Mutante, to 139.261 µg/g FER, in Etiopia MP 3.

Once again, FER has increased in relation to the H_2O fraction and, at a larger extent, to the EtOH fraction. The concentration of FER in the NaOH fraction was about 3 to 4 times higher than in the H_2O fraction. The highest content of FER (139.26 ± 20.77 µg/g) was observed in winter 2014 of Etiopia MP 3 (Figure 26).

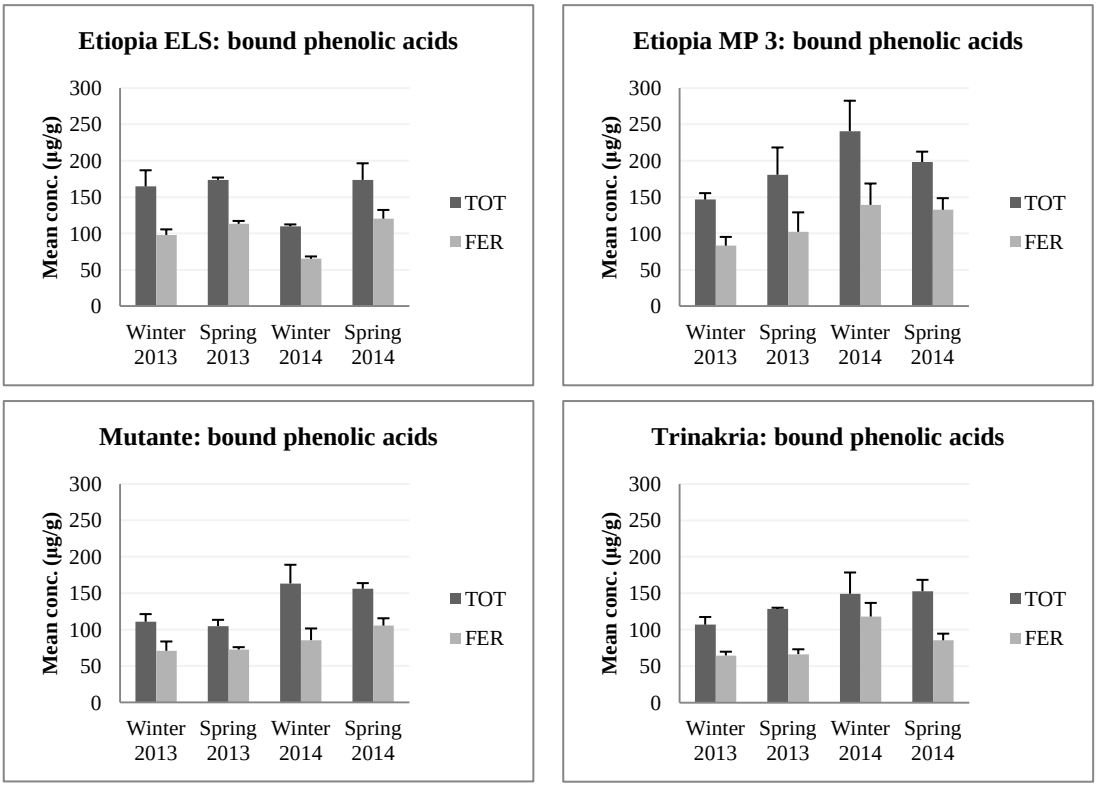


Figure 26: Mean concentration of the total (TOT) phenolic acids bound to structures soluble in NaOH alongside to the mean concentration of ferulic acid (FER) found in the four cultivars under study, in winter and spring of 2013 and 2014 (mean µg/g ± SD, α=0.05).

Total bound PAs of Mutante and Trinakria have increased from winter 2013 to winter 2014 and from spring 2013 to spring 2014, while for Etiopia MP 3 their

content have increased only from winter 2013 to winter 2014 (Figure 27) . Total bound PAs of Etiopia ELS tended to decrease from winter 2013 to winter 2014. Overall, Mutante and Trinakria presented a quite similar tendency among years and sowing seasons. The highest amount of total bound PAs of 240.55 ± 29.62 µg/g was observed for Etiopia MP 3 in winter 2014 (Figure 27).

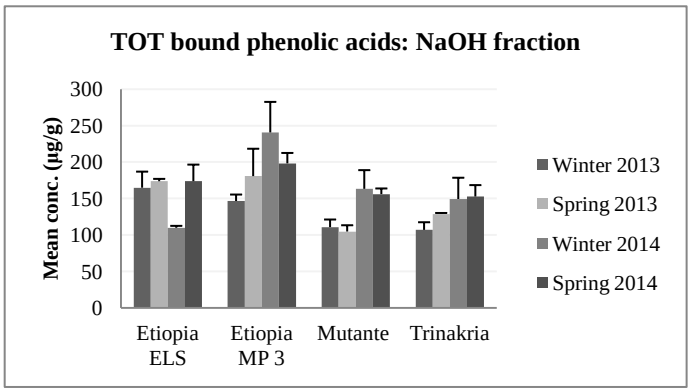


Figure 27: Mean concentration of the total (TOT) phenolic acids bound to structures soluble in NaOH present in the four cultivars under study, in winter and spring of 2013 and 2014(mean µg/g ± SD, α=0.05).

Etiopia ELS had a significantly higher concentration of 4-OHB in 2013 relative to 2014 ($p<0.05$) (Table 8). Winter sowing caused a significant increase on the concentration of CAT, while the same effect happened in spring for SYR and FER. The interaction 2013 winter provided for significantly higher contents of CAT and p-COU; 2014 spring and 2013 spring for FER; and 2014 winter provided for the significantly lower amount of TOT bound phenolic acids relatively to the other interactions.

For Etiopia MP 3, 2014 contributed to significantly higher contents of VAN, SYR, CAF and FER, on the contrary to p-COU, which contribution was done in 2013 (Table 8). Concentrations of PRO, CAT and 4-OHB were significantly higher for winter sowing compared to durum wheat sown in spring. Yet, spring produced significantly higher contents of SYR and p-COU (both $p<0.05$). The interaction year x sowing season had different effects on the content of PAs, having significantly higher concentrations with the interactions that follow: VAN in 2014

spring and 2014 winter, SYR in 2014 spring, di-OHB in 2013 spring and 2013 winter, p-COU in 2013 spring and SIN in 2014 winter.

The year 2014 was the most productive for Mutante, once it has provided for a significant increase on the concentrations of 4-OHB, VAN, SYR, di-OHB, FER and TOT bound phenolic acids (Table 8). The year 2013 has provided with the same effect only on the concentration of p-COU. Winter sowing produced a significantly higher concentration of di-OHB. The interactions 2014 spring and 2014 winter provoked a significant increase on the concentration of, respectively, 4-OHB ($p<0.001$) and di-OHB ($p<0.05$); and 2014 winter and 2014 spring on TOT bound phenolic acids.

For Trinakria, 2014 was also a better year providing a significantly increased production of FER and TOT bound phenolic acids (Table 8). While 2013 produced significantly higher content of SIN. In spring, the content of PRO and di-OHB was significantly increased in relation to winter sowing. The interaction 2014 spring provoked a significant increase on the concentration of PRO and di-OHB, 2013 spring on SYR and p-COU, 2014 winter on FER, 2013 spring and 2014 winter on CAF, and 2013 spring and 2013 winter on SIN.

Table 8: Statistical analysis of the content of phenolic acids bound to structures soluble in NaOH, present in four cultivars of durum wheat sown in winter and spring of 2013 and 2014.

			Mean concentration (µg/g)										
Cultivar	year	season	PRO	CAT	4-OHB	VAN	SYR	di-OHB	CAF	p-COU	SIN	FER	TOT
Etiopia ELS	2013	Winter	1.44	5.01 b	0.80	6.02	0.76	31.65	1.65	2.71 b	16.82	97.89 b	164.75 b
		Spring	1.41	1.81 a	0.86	3.02	2.68	32.34	0.66	nd a	17.63	113.24 bc	173.65 b
	2014	Winter	0.85	2.93 a	0.45	2.35	2.02	24.10	0.46	nd a	11.50	65.14 a	109.81 a
		Spring	0.94	1.43 a	0.30	2.89	2.56	30.27	0.52	nd a	14.40	120.36 c	173.68 b
		year	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
		sowing	ns	**	ns	ns	*	ns	ns	ns	ns	*	ns
		year x sowing	ns	*	ns	ns	ns	ns	ns	*	ns	**	*
Etiopia MP 3	2013	Winter	1.81	5.12	2.34	2.58 a	1.59 a	36.50 bc	0.59	2.04 a	10.74 a	83.21	146.53
		Spring	0.98	3.37	0.34	3.31 a	2.43 a	51.45 cd	0.69	2.77 b	13.08 a	102.26	180.67
	2014	Winter	1.79	5.22	1.64	5.20 b	2.53 a	57.29 d	1.12	1.77 a	24.73 b	139.26	240.55
		Spring	0.13	2.59	1.08	5.30 b	7.71 b	31.18 a	0.89	2.02 a	14.13 a	132.45	197.48
		year	ns	ns	ns	**	*	ns	*	*	ns	*	ns
		sowing	*	*	*	ns	*	ns	ns	*	ns	ns	ns
		year x sowing	ns	ns	ns	*	**	*	ns	*	*	ns	ns
Mutante	2013	Winter	0.93	3.20	0.36 b	0.71	0.78	24.69 a	0.30	1.42	7.48	70.80	110.67 a
		Spring	0.87	1.21	0.15 a	1.48	1.39	18.93 a	0.38	1.93	5.74	72.63	104.70 a
	2014	Winter	1.25	2.07	1.04 c	3.28	2.93	59.91 b	0.27	0.59	6.45	85.42	163.21 b
		Spring	1.20	2.31	1.25 d	3.13	3.26	29.38 a	0.17	1.08	8.86	105.38	156.01 b
		year	ns	ns	***	*	*	*	ns	*	ns	*	**
		sowing	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
		year x sowing	ns	ns	***	ns	ns	*	ns	ns	ns	ns	*
Trinakria	2013	Winter	0.46 a	4.78	0.44	1.46	1.51 a	19.27 a	0.36 a	nd a	13.97 b	64.67 a	106.93
		Spring	0.70 a	4.74	0.88	1.90	6.46 b	30.06 a	0.47 ab	0.33 b	16.80 b	66.43 a	128.77
	2014	Winter	0.46 a	2.59	0.25	1.93	1.50 a	16.03 a	0.68 b	nd a	7.84 a	118.02 b	149.29
		Spring	1.33 b	3.52	0.30	1.48	1.14 a	53.05 b	0.27 a	nd a	6.14 a	85.46 a	152.68
		year	ns	ns	ns	ns	ns	ns	ns	ns	**	*	*
		sowing	*	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
		year x sowing	*	ns	ns	ns	*	*	*	*	**	*	ns

Significance (n=3) is shown as * for p<0.05, ** for p<0.01, and *** for p<0.001, ns when means are not significantly different. nd, not detected.

3.2.2.4. TOT phenolic acids among varieties

The total free and total bound phenolic acids of the EtOH, H_2O and NaOH fractions found in Etiopia ELS, Etiopia MP 3, Mutante and Trinakria, sown in winter and spring of 2013 and 2014, are evidenced in Table 9. In addition, the total bound phenolic acids (TOT bound) as the sum of the PAs of all fractions is shown. Concentration of total phenolic acids increase from fraction to fraction.

A tendency of a lower amount of TOT bound phenolic acids in the year 2013 relative to 2014 was observed for all the varieties, except for Etiopia ELS.

The effect of Etiopia MP 3 genotype was the most important for the production of PAs, containing significantly higher concentrations of TOT phenolic acids in relation to other varieties in the H_2O fraction ($p<0.001$), NaOH fraction ($p<0.01$) and in the TOT bound phenolic acids ($p<0.001$) (Table 9). The year that provided for significantly higher concentrations of PAs was 2014 for the fractions EtOH free ($p<0.01$), NaOH ($p<0.05$) and TOT bound phenolic acids ($p<0.05$).

Statistically, the interaction (variety *x* year) of Etiopia MP 3 with 2014 contributed for significantly higher amounts of phenolic acids in all the fractions and TOT bound phenolic acids (Table 9). The same for the interaction of variety per sowing, Etiopia MP 3 sown in winter produced the highest values of PAs in NaOH and TOT bound fractions. In addition, the results obtained for Etiopia MP 3 sown in winter were in all cases statistically similar to those of Etiopia MP 3 sown in spring.

The interaction variety *x* year *x* sowing resulted in statistical differences among all the fractions and TOT bound PAs, however many similarities were found. The highest and lowest content of PAs were seen, respectively, for: Etiopia MP 3 in winter 2014 and Mutante in winter 2013, in the EtOH fraction of free PAs ($p<0.01$); Mutante in spring 2014 and Mutante in winter 2013, in the EtOH fraction of bound PAs ($p<0.001$); Etiopia MP 3 in spring 2014 and Trinakria in spring 2014, in the H_2O fraction ($p<0.001$); Etiopia MP 3 in winter 2014 and Mutante in spring 2013, in the NaOH fraction ($p<0.001$); Etiopia MP 3 in winter 2014 and Trinakria in winter 2013, for the TOT bound phenolic acids ($p<0.001$).

The best interaction of genotype with environmental conditions that resulted in a bigger production of phenolic acids has been brought out by Etiopia MP 3 sown

in winter of 2014. In spring of 2014 the production of total bound PAs was lower, but still statistically similar to that of winter 2014.

Bound phenolic acids of the EtOH, H_2O and NaOH fractions, contribute with respectively 5.1%, 35.7% and 59.2% for the total bound phenolic acids (see Table 9). Free PAs constitute only a small part of the total bound phenolic acids (4.9%), yet similarly to the bound PAs of the same ethanolic fraction.

Table 9: Total free (EtOH free) and bound phenolic acids of the ethanol (EtOH bound), water (H_2O) and sodium hydroxide (NaOH) fractions, determined in the four cultivars of durum wheat sown in winter and spring of 2013 and 2014. Total bound phenolic acids (TOT bound) of all fractions are shown.

Cultivar	Year	Season	**EtOH free (µg/g)**		**EtOH bound (µg/g)**		**H_2O (µg/g)**		**NaOH (µg/g)**		**º TOT bound (µg/g)**	
Etiopia ELS	2013	Winter	15,250	def	6,989	abc	106,779	de	164,751	def	278,518	cde
		Spring	15,087	def	9,560	abcd	123,627	f	173,653	ef	306,839	e
	2014	Winter	12,535	bcdef	10,071	abcd	108,475	def	109,808	abc	228,354	abc
		Spring	14,775	cdef	17,499	ef	101,571	d	173,683	ef	292,753	de
Etiopia MP 3	2013	Winter	5,726	ab	15,392	def	119,772	ef	146,526	abcde	281,690	de
		Spring	7,740	abcd	7,570	abc	101,165	d	180,674	ef	289,408	de
	2014	Winter	19,947	f	20,781	f	144,875	g	240,548	g	406,204	f
		Spring	18,867	f	19,541	f	153,040	g	198,167	fg	370,748	f
Mutante	2013	Winter	2,246	a	3,504	a	70,634	bc	110,670	abc	184,809	a
		Spring	8,818	abcde	11,270	bcde	75,111	c	104,704	a	191,085	ab
	2014	Winter	15,175	def	12,191	cde	65,925	bc	163,211	def	241,328	bcd
		Spring	15,652	ef	20,934	f	63,728	bc	156,015	def	240,676	bcd
Trinakria	2013	Winter	10,711	bcde	20,594	f	56,279	ab	106,933	ab	183,806	a
		Spring	7,283	abc	14,110	cdef	66,226	bc	128,773	abcd	209,109	ab
	2014	Winter	11,146	bcde	7,272	abc	70,456	bc	149,288	bcde	227,016	abc
		Spring	6,394	ab	4,421	ab	44,117	a	152,680	cde	201,219	ab
Statistical factors		variety	ns		ns		***		**		***	
		year	**		ns		ns		*		*	
		sowing	ns		ns		ns		ns		ns	
		var x year	***		**		***		***		***	
		var x sowing	ns		ns		***		*		***	
		year x sowing	ns		ns		ns		ns		ns	
		var x year x sowing	**		***		***		***		***	

Significance (n=3) is shown as * for p<0.05, ** for p<0.01, and *** for p<0.001, ns when means are not significantly different.
º TOT bound is calculated as the sum of phenolic acids from all bound PAs-fractions (EtOH bound, H_2O and NaOH)

3.3. Determination of resistant and non-resistant starch

3.3.1. Resistant starch

Quantification of resistant starch (RS) in the four cultivars of durum wheat sown in winter and spring of 2013 and 2014 is evidenced in Table 10 (see mean ± SD in Table 16, in Appendix).

Etiopia ELS in 2014 produced the highest quantity of resistant starch among all the varieties, in winter with 0.52 g Glc/100g of flour and in spring with 0.47 g Glc/100g flour (Figure 28, Table 10). Very close to these values was RS produced by Etiopia MP 3 in winter of 2013, 0.39 g Glc/100g. The poorest genotype on the presence of RS was Trinakria sowed in spring of 2014 with 0.04 g Glc/100g.

Winter contributed for a significantly higher production of RS (winter mean 0.28 g Glc/100g vs. spring mean 0.17 g Glc/100g; $p<0.05$).

The interaction (variety *x* year) of Etiopia ELS with 2014 resulted in a significant increase on the content of RS (mean 0.50 g Glc/100g, $p<0.01$). The interaction (variety *x* sowing) of Etiopia ELS with winter sowing contained the highest content of RS (0.52 g Glc/100g) and Trinakria in spring the lowest (0.04 g Glc/100g) ($p<0.05$). The interaction (year *x* sowing) of 2014 winter showed a higher content of RS (mean 0.28g Glc/100g), which was higher than in 2014 spring and 2013 winter, and significantly higher than 2013 spring (mean 0.11g Glc/100g, $p<0.05$). The interaction (variety *x* year *x* sowing) of Etiopia ELS with the winter sowing of 2014 provided for higher content of RS, with 0.52g Glc/100g.

Etiopia ELS sowed in 2014 winter produced statistically similar amounts of RS as in 2014 spring (mean 0.47g Glc/100g), and also similar amounts relatively to Etiopia MP 3 sowed in 2013 winter (mean 0.39g Glc/100g).

Mutante in winter 2013 and Trinakria in winter 2014 with, respectively, 0.31 g Glc/100g and 0.30 g Glc/100g, produced statistically correlated amounts of RS.

All the other variety *x* year *x* sowing combinations produced significantly lower RS compared to Etiopia ELS sowed in both seasons of 2014 ($p<0.001$) (Table 10).

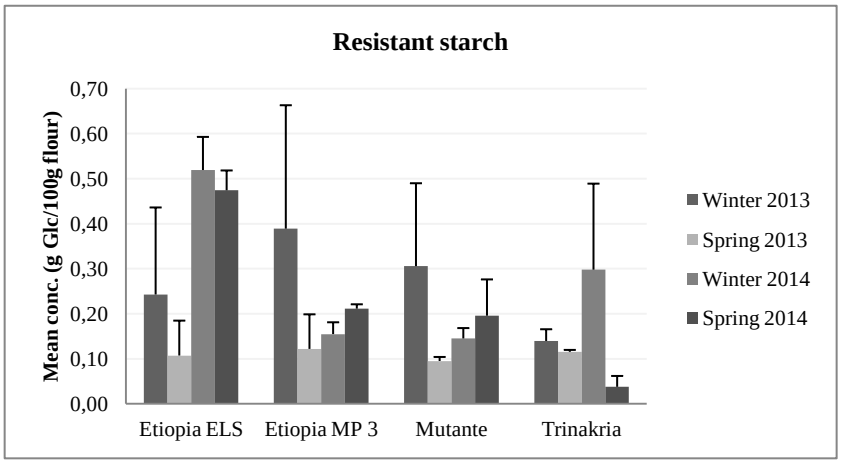


Figure 28: Content of resistant starch found in four durum wheat cultivars, sown in winter and spring of 2013 and 2014 (mean in gram of glucose per 100g of durum wheat flour ± SD; axis range: 0.0-0.7 g Glc/100g).

3.3.2. Non-resistant starch (solubilized starch)

Quantification of non-resistant starch (NRS) in the four cultivars of durum wheat sown in winter and spring of 2013 and 2014 is evidenced in Table 10 (see mean ± SD in Table 16, in Appendix).

The highest content of NRS was obtained by Etiopia ELS in winter 2014 (38.11g Glc/100g) and spring 2014 (41.08 g Glc/100g), which is in accordance to the highest content of RS found also in this genotype (Figure 29, Table 10).

Etiopia MP 3 has gotten a high amount of NRS of 38.93g Glc/100g in winter 2014, statistically similar to Etiopia ELS in winter and spring of 2014 and to its own cultivation on spring 2013, with 31.33 g Glc/100g. Yet, all the other combinations of variety *x* year *x* season sowing had significantly lower NRS compared to that of Etiopia ELS sowed in 2014 winter ($p<0.01$).

The interaction (variety *x* year) of Etiopia ELS with the year 2014 resulted in a significant increase on the content of NRS (39.6g Glc/100g, $p<0.01$), over the remaining homogeneous groups. The lowest amount of NRS of 21.6 g Glc/100g was obtained for Etiopia MP 3 sowed in 2014 spring, yet statistically correlated with the amount obtained for Mutante, Trinakria and Etiopia ELS sowed in 2013.

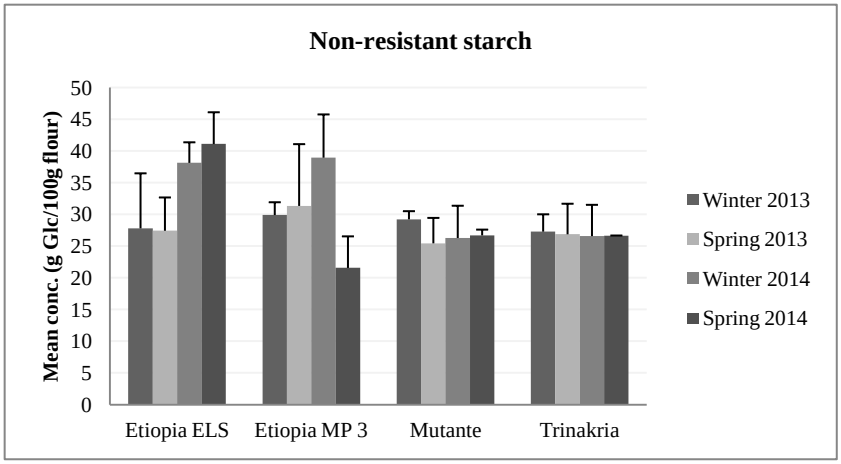


Figure 29: Content of non-resistant starch found in four durum wheat cultivars, sown in winter and spring of 2013 and 2014 (mean in gram of glucose per 100g of durum wheat flour ± SD axis range: 0-50 g Glc/100g).

Table 10: Statistical results for the content of resistant (RS) and non-resistant starch (NRS) of four durum wheat cultivars sown in winter and spring of 2013 and 2014.

			Mean concentration (g Glc/100g flour)			
Cultivar	Year	Season	**RS**	v *x* y *x* s	**NRS**	v *x* y *x* s
Etiopia ELS	2013	Winter	0.24	bcde	27.79	ab
		Spring	0.11	abc	27.41	ab
	2014	Winter	0.52	g	38.11	cde
		Spring	0.47	fg	41.08	e
Etiopia MP 3	2013	Winter	0.39	efg	29.89	abc
		Spring	0.12	abcd	31.33	bcd
	2014	Winter	0.15	abcd	38.93	de
		Spring	0.21	abcde	21.55	a
Mutante	2013	Winter	0.31	def	29.21	ab
		Spring	0.10	ab	25.43	ab
	2014	Winter	0.15	abcd	26.27	ab
		Spring	0.20	abcd	26.69	ab
Trinakria	2013	Winter	0.14	abcd	27.28	ab
		Spring	0.12	abcd	26.86	ab
	2014	Winter	0.30	cdef	26.57	ab
		Spring	0.04	a	26.60	ab
		variety	*		*	
		year	ns		ns	
		sowing	*		ns	
		variety *x* year	**		**	
		variety *x* sowing	*		ns	
		year *x* sowing	*		ns	
variety x year x sowing (v *x* y *x* s)			***		**	

Significance (n=3) is shown as * for p<0.05, ** for p<0.01, and *** for p<0.001, ns when differences are not significant.

3.4. Discriminant Analysis

The discriminant analysis indicated that both the polyphenols and the starch contributed to the differentiation among the four durum wheat cultivars grown in winter and spring of 2013 and 2014 (Table 11). The Wilks' lambda of 0.05515 (significant at $p<0.001$) and F value (10.99) indicate the somewhat high discriminatory power of the applied model to classify durum wheat genotypes based on the free and total bound PAs and the resistant and non-resistant starch. Wilk's lambda number means that 5.5% of the variance was not explained by the group differences. Eigenvalues gives information about the effectiveness of the discriminant functions (the higher, the most effective) in distinguishing between groups: 7.23 for function 1 and 1.17 for function 2.

Figure 30 shows the scatterplot of the wheat genotypes defined by two canonical functions, according to the concentration of total free and total bound PAs of each fraction, and the content of resistant and non-resistant starch. Function 1 (the most competent in differentiating the groups, DF1) and Function 2 (DF2) explained, cumulatively, 85.9% and 99.8% of the variability. Score plot shows the relationships among different samples.

Table 11 shows the relationships among variables. The interpretation should be focused on the variables with larger absolute loadings and standardized coefficients > 1 are interpreted as defining the variables. TOT bound phenolic acids of the H_2O fraction were positioned at the farthest positive value of the first DF and thus dominated this function. Bound phenolic acids of the EtOH and NaOH fractions were found at the positive end of the second DF. They were negatively correlated with the starch components and the free phenolic acids. The variables that were not well explained by the discriminatory analysis might be more susceptible to environmental effects.

Once all the varieties of durum wheat were grown at the same location, over the same environmental conditions, the variables with high genotypic variance (less affected by the environment) are better described by the canonical functions than variables with high environmental variance[79].

Table 11: Discriminatory power of the discriminant analysis using 16 sub-groups (two season sowing x two years x four varieties). The canonical variables of the discriminant functions are listed and the respective standardized coefficients given.

Variable		Standardised coefficient	
		DF1	DF2
TOT free PAs, EtOH		-0.76	-1.64
TOT bound PAs, EtOH		0.17	1.14
TOT bound PAs, H_2O		1.08	-0.06
TOT bound PAs, NaOH		0.54	0.92
Resistant starch		0.09	-0.21
Non-resistant starch		0.3	-0.49
	Eigenvalues	7.23	1.17
	Cumulative explained variance (%)	85.9	99.82
	Trait into the model (n)	16	
	Wilks'λ	0.05515	
	F	10.99	
	P	0.0000	

The scatter plot diagram of the canonical coefficients (Figure 30) shows that the distances between the genotypes were greater than the distances between the winter-sown and spring-sown plants of the same genotype. Particularly**,** Trinakria and its genetically modified pair Mutante clustered together, which is in agreement with their genotypic affinity, indicating they have a similar response over the same environmental conditions (Figure 30). Etiopia ELS and Etiopia MP 3 clustered between themselves in about half the area, and do not cluster with Trinakria or Mutante. The two Ethiopian lines grouped towards the more positive region of the first canonical function, with ELS genotype being in the more positive side of the second canonical function and MP 3 genotype in the more negative side.

The scatter diagram for the first two canonical variables highlights that biodiversity has the most significant role in determination of the healthy quality of the grain. Of the different compounds used in the discriminant analysis, in terms of their effectiveness in separating the genotypes, the total bound PAs of the H_2O fraction had the most significant role. The two Etiopia genotypes had the greatest amount of total bound PAs of the H_2O fraction compared to the other two genotypes.

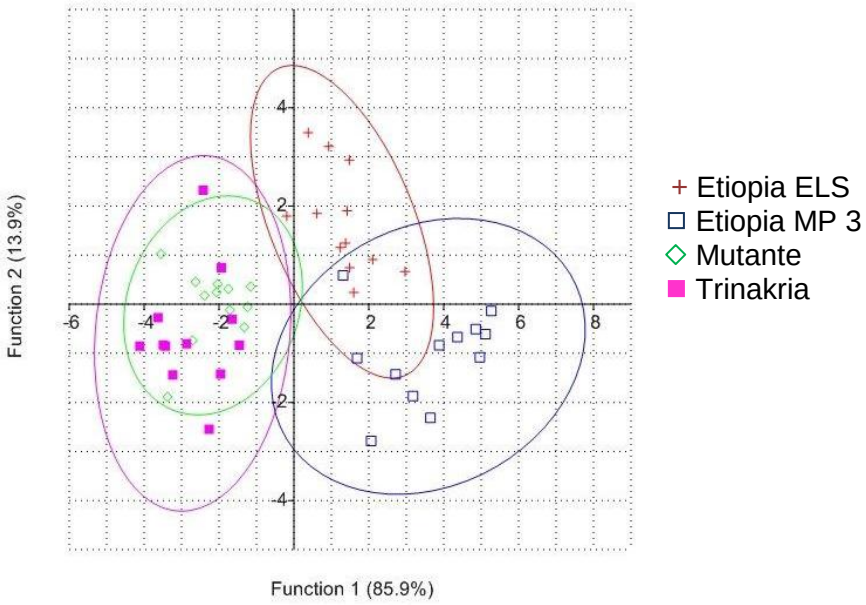


Figure 30: Canonical discriminant analysis of four durum wheat varieties grown in winter and spring of 2013 and 2014, according to the concentration of total free and total bound PAs of each fraction, and the content of resistant and non-resistant starch.

4. Discussion

Durum wheat has always been predominantly used for the production of pasta, especially in the Italian culture. Today is well known the health promoting effects of secondary plant metabolites (e.g. phenolic acids) and dietary fibre (e.g. resistant starch), which are found in durum wheat. If one includes the bran and aleurone layer, the whole durum wheat flour comprehends a higher amount of these compounds.

Global climate change and consumers' increasing interest on healthy compounds have pushed research towards evaluations on wheat to be preserved for the future. Wheat cultivars rich in healthy compounds and the most resistant to environmental and soil conditions must be protected. For this reason we wanted to characterize four durum wheat cultivars based on the content of phenolic acids, resistant starch and non-resistant starch, taking in account the growing environmental conditions and the genotype.

The methodology used in this study, using different solvent extracts in serie to determine the phenolic acids content is, to our knowledge, innovative.

4.1. Phenolic acids

Even though methodologies for the determination of phenolic acids found in literature were diverse from the one taken in the current study, results of others have been used for comparison.

Free phenolic acids reported in Canadian wheat (*Triticum turgidum* L. var. *durum* and *Triticum aestivum* L.) were higher relatively to the four durum wheat cultivars under study even though 4-OHB, VAN and CAF of one of the species was detected only in our samples[80]. In the same study, bound phenolic acids were also relatively higher[80].

The concentration of free FER found in Trinakria grown in winter of 2014 (4.07 and 2.40 μg/g) was observed in literature before in bran[81]. The same as for CAF with 1.78 μg/g found in literature[81].

Durum wheat whole grains, analyzed under HEALTHGRAIN program for the content of free and bound phenolic acids, showed 13±2 μg/g of free and 418±51 μg/g of bound[82]. Free phenolic acids were in accordance with that of the four cultivars in our study. Bound PAs were also equivalent especially for Etiopia MP 3

in 2014, while in the other cases were slightly lower. In the same study single phenolic acids were identified, bound 4-OHB, bound VAN, free and bound di-hydroxybenzoic acids (2,4-dihydroxybenzoic acid), bound SIN, and free FER were similarly quantified as for the durum wheat varieties of the present study. Another HEALTHGRAIN diversity screens on durum wheat demonstrated a composition of free phenolic acids of 11 µg/g and bound PAs of 485-492 µg/g, the former in accordance to our results and the latter 2-fold higher in most cases[79,83].

In a recent study, where SPE methodology was also used, concentrations of free and bound 4-OHB, VAN, SYR, SIN and FER, free CAF and p-COU, were rather similar to that of the present study[84].

The amount of 4-OHB (1.85±0.29 µg/g), CAT (4.47±0.17 µg/g), VAN (3.12±0.14 µg/g), p-COU (2.5±0.05 µg/g), FER (62.31±2.38 µg/g) on durum semolina flour of a previous study are comparable with that of the H_2O and NaOH fractions in the present study, although FER was generally higher in our samples[85].

Bound SYR found in the H_2O and NaOH fraction was similar to the concentration of SYR found in common wheat and its presence showed to be higher in the H_2O fraction, also observed previously[86]. The same for the presence of FER (soluble conjugated) in common wheat, also similar to that of the H_2O fraction[86].

FER is the major compound in the NaOH fraction, which indicates that FER is bounded to cell wall structures of high molecular weight. Such abundance has been previously reported in literature[80,82,84–86].

4.1.1. Effect of climatic conditions of growth

During the grain filling period of winter sown wheat (in May) rainfalls were similar between years, however the temperature in 2013 was higher than in 2014 of about 7 ºC. The grain filling period of spring sown wheat (in June) was less rainy and hotter in 2013 than 2014. In general, 2013 was warmer than 2014. The rainfalls tendency was comparable in both years, except in April, which was rainier in 2014.

The production of phenolic acids tended to increase by the effect of winter sowing and the year 2014. Additionally, the genotype, particularly Etiopia MP 3, had a great effect on the production of PAs. The amount of phenolic acids found on durum wheat might have been influenced by the cooler year of 2014 relative to 2013. Rainfalls were similar in both years for the winter grain filling period, however the pre-filling period of the grain was rainier in 2014 (April 2014, Figure 12 and Figure 13) which might have prompted to higher amounts of PAs. All in all, these results make us believe of a relationship between the production of phenolic acids and the pre- and actual filling period of the grain. The tendency shows that a rainier pre-filling period and a cooler filling period (10-15ºC mean T) of the grain leads to higher contents of PAs on durum wheat whole flours.

A rainier pre-filling period and a cooler filling period of the durum wheat grains leaded to significantly higher content of total polyphenols (mainly include phenolic acids and flavonoids) in previous studies[87]. However at the same time, it leaded to significantly lower content of free polyphenols.

The biosynthesis and accumulation of phenolic compounds during the kernel development has shown to be dependent on the genotype and environmental conditions[87].

4.2. Resistant and Non-resistant starch

Human diet consists of more than 50% of starch-based foods[28]. The amount of resistant starch in starch-based foods is therefore important to provide the most benefitial health effects.

Several studies on durum wheat varieties reported a content of resistant starch ranging 0.30-0.7 g/100g, which is comparable with that of the four cultivars of durum wheat in this study[28,87–89]. Another studies showed that hard and soft varieties of wheat (*T. aestivum* L.) contained between 0.20% and 0.55% of resistant starch[90,91]. These studies are in accordance with that of Etiopia ELS in winter 2014 (0.52 g Glc/100g flour), Etiopia MP 3 in winter 2013 (0.39±0.27 g Glc/100g flour), Mutante in winter 2013 (0.31±0.18 g Glc/100g flour), and Trinakria in winter 2014 (0.30±0.19 g Glc/100g flour). Relatively to the conventional and waxy durum wheat grains of the cultivar Svevo, widely used in the manufacture of

traditional wheat products, resistant starch content was relatively higher: 0.9% dm and 1.2% dm, respectively[92].

Season sowing had a significant effect on RS content, previously observed by others[87]. Winter was the most effective on the production of resistant starch. The year in contrast did not have a significant affect.

A recent study on three durum wheat cultivars verified a content of non-resistant starch between 40.8 % and 50.5 % dw, as good as that of Etiopia ELS (winter 2014, 38.11±3.24 g Glc/100g flour; spring 2014, 41.08±5.01 g Glc/100g flour) and Etiopia MP 3 (winter 2014, 38.93±6.82 g Glc/100g flour; spring 2013, 31.33±9.74 g Glc/100g flour)[88].

5. Conclusions and Future perspectives

Whole grain cereals are recognized sources of biologically active components that promote health. The present study indicates that durum wheat is a valuable source of these constituents, explicitly resistant starch (part of dietary fibre), starch and phenolic acids (part of polyphenols).

HPLC-DAD analysis enabled the identification and quantification of protocatechuic acid, catechin, 4-hydroxybenzoic, vanillic acid, syringic acid, di-hydroxybenzoic acids, caffeic acid, *p*-coumaric acid, sinapic acid and ferulic acid, in whole durum wheat flours under study. The SPE methodology offers high recovery and precision of phenolic acids present in wheat flour, and might be used in laboratory routine analysis of wheat and wheat based products. FER was the major compound in the NaOH fraction, which indicates that FER is bounded to cell wall structures of high molecular weight. Di-hydroxybenzoic acids concentration was comparable with that of FER in the EtOH free, EtOH bound and H_2O bound fractions.

The amount and character of those bioactive substances in grain depend upon the genotype and the environmental conditions of growth. Environmental factors have a strong impact on the production secondary metabolites in plants, confirmed by the modifications of the content of PAs in the four varieties over the two seasons each year.

The changes on the concentration of healthy compounds as induced by the climatic conditions of plant growth, coupled with the interaction of genotype, helped to understand durum wheat responses to abiotic and genotypic effects. During current climatic changes on planet Earth, biodiversity is assuming a more important role for the maintenance of human health and survival in the future.

The production of phenolic acids tended to increase by the effect of winter sowing and the year 2014. The amount of phenolic acids found on durum wheat might have been influenced by the cooler year of 2014 relative to 2013. The tendency shows that a rainier pre-filling period and a cooler filling period (10-15ºC mean T) of the grain leads to higher contents of PAs on durum wheat whole flours.

Additionally, the genotype, particularly Etiopia MP 3, had a great effect on the production of PAs. Its profile showed, in all the fractions, higher PAs content in 2014 relatively to 2013, which indicates 2014 as more productive for this specie.

Discriminant analysis have demonstrated correlations between lines from the same genotypic origin (Mutante and Trinakria) or with genotypic affinity (Etiopia ELS and MP 3), confirming that for wheat the biodiversity is more important in the determination of healthy compounds compared to the environmental effect. Genotype has the greatest effect on the production of PAs.

HPLC-RI analysis for the determination of resistant starch is a more sensitive and accurate technology than the frequently used GODOP reagent, which however is a faster methodology. Highest contents of resistant starch of 0.3-0.5 g Glc/100g flour were obtained in winter season, therefore the most effective sowing season for the production of this structures. The year did not have a significant effect. The results were not conclusive when correlating environmental conditions of growth with the content of resistant starch.

Etiopia ELS and Etiopia MP 3 genotypes provided the highest production of PAs and RS. These genotypes may be reasonably taken in account for usage in breeding programs, because of the content on those bioactive substances.

Above all, it is clearly necessary to have more years of study to adjudicate the effects of the environmental conditions on the concentration of healthy compounds. In addition increased representative samples would add value to future studies.

Wheat based products higher in phenolic acids and resistant starch might lead to a diet richer in bioactive substances that promote health. Nevertheless, further research on other antioxidant compounds (e.g. tocotrienols, tocopherols, vitamin A, carotenoids, glutathione) will be necessary to better assess the health benefits of consuming wheat or cereal-based foods. Moreover, it would be important to evaluate plant cereals genotypic disposition, growing environmental conditions, nutritional qualities, and technological potential together, in order to have an assessment that is more precise and rigorous. With this, a database could be built to make a selection of the best varieties for each specific environment, so that they could be preserved for the future.

6. Bibliography

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Appendix

Table 12: Concentration (mean µg/g ± SD) of free phenolic acids present in four cultivars of durum wheat, sown in winter and spring of 2013 and 2014.

Cultivar	Year	Sowing	**PRO**	SD	**CAT**	SD	**4-OHB**	SD	**VAN**	SD	**SYR**	SD	**di-OHB**	SD	**CAF**	SD	**p-COU**	SD	**SIN**	SD	**FER**	SD	**TOT**	SD
Etiopia ELS	2013	Winter	0.63	0.22	nd	-	1.80	0.42	0.25	0.08	0.17	0.04	12.10	3.67	0.06	0.01	nd	-	0.08	0.03	0.16	0.07	15.25	3.90
	2013	Spring	0.33	0.11	10.32	3.25	1.43	0.10	0.01	0.00	1.17	0.39	0.78	0.26	0.05	0.03	nd	-	nd	-	0.99	0.32	15.09	3.63
	2014	Winter	0.19	0.03	4.35	0.93	0.21	0.04	0.18	0.08	0.19	0.04	7.20	1.50	0.07	0.02	nd	-	0.06	0.01	0.10	0.00	12.53	0.48
	2014	Spring	0.19	0.04	6.24	2.69	0.11	0.04	0.46	0.15	0.15	0.00	7.38	3.28	0.07	0.01	nd	-	0.06	0.01	0.13	0.01	14.77	6.13
Etiopia MP 3	2013	Winter	0.14	0.05	1.97	0.80	0.29	0.02	0.33	0.07	0.09	0.02	2.17	0.73	0.37	0.13	0.12	0.00	nd	-	0.25	0.07	5.73	1.52
	2013	Spring	0.40	0.15	2.64	0.43	0.83	0.19	0.64	0.20	0.14	0.03	2.48	0.83	0.08	0.02	nd	-	0.13	0.01	0.40	0.19	7.74	0.94
	2014	Winter	0.08	0.03	10.67	3.24	3.31	1.03	0.58	0.19	0.24	0.08	4.32	1.44	0.06	0.02	nd	-	0.13	0.01	0.57	0.03	19.95	3.30
	2014	Spring	0.26	0.08	5.71	2.20	4.44	0.18	0.46	0.00	0.56	0.02	6.35	0.82	0.26	0.01	0.50	0.06	0.13	0.01	0.20	0.04	18.87	3.02
Mutante	2013	Winter	0.08	0.04	0.39	0.06	0.21	0.05	0.32	0.20	0.12	0.04	1.03	0.34	0.04	0.02	nd	-	nd	-	0.05	0.00	2.25	0.45
	2013	Spring	0.17	0.08	0.27	0.01	0.56	0.01	2.04	0.02	1.05	0.10	1.68	0.30	0.38	0.11	nd	-	0.20	0.07	2.49	0.81	8.82	0.68
	2014	Winter	0.83	0.20	2.85	0.67	0.95	0.39	0.22	0.07	0.37	0.08	7.32	1.68	2.49	0.79	nd	-	nd	-	0.16	0.03	15.17	0.34
	2014	Spring	1.05	0.16	5.84	1.34	1.17	0.02	0.27	0.09	0.31	0.07	4.10	1.37	0.74	0.25	0.17	0.05	0.32	0.07	1.70	0.50	15.65	0.59
Trinakria	2013	Winter	0.07	0.02	5.11	1.55	0.12	0.02	0.43	0.10	0.29	0.09	4.46	1.10	0.10	0.00	nd	-	0.04	0.02	0.10	0.01	10.71	2.37
	2013	Spring	0.71	0.24	0.93	0.28	0.57	0.21	0.38	0.14	0.66	0.15	3.76	0.96	0.10	0.02	0.06	0.02	0.02	0.00	0.10	0.02	7.28	0.39
	2014	Winter	0.49	0.13	2.85	0.54	0.05	0.02	0.52	0.14	0.15	0.00	2.53	0.30	0.48	0.14	nd	-	0.03	0.01	4.07	1.30	11.15	1.92
	2014	Spring	0.24	0.01	0.93	0.03	0.43	0.13	0.39	0.07	0.11	0.02	2.01	0.22	0.10	0.03	nd	-	nd	-	2.17	0.70	6.39	0.73

PRO, protocatechuic acid; CAT, catechin; 4-OHB, 4-hydroxybenzoic acid; VAN, vanillic acid; SYR, syringic acid; di-OHB, di-hydroxybenzoic acids; CAF, caffeic acid; p-COU, p-coumaric acid; SIN, sinapic acid; FER, ferulic acid
SD, standard deviation. nd, not detected.

Table 13: Concentration (mean µg/g ± SD) of phenolic acids bound to structures soluble in EtOH, present in four cultivars of durum wheat sown in winter and spring of 2013 and 2014.

Cultivar	year	sowing	**PRO**	SD	**CAT**	SD	**4-OHB**	SD	**VAN**	SD	**SYR**	SD	**di-OHB**	SD	**CAF**	SD	**p-COU**	SD	**SIN**	SD	**FER**	SD	**TOT**	SD
Etiopia ELS	2013	Winter	0.11	0.04	0.69	0.23	0.18	0.02	0.41	0.18	0.52	0.13	2.85	0.65	0.09	0.04	0.13	0.04	0.39	0.05	1.62	0.09	6.99	0.36
	2013	Spring	0.35	0.11	0.55	0.04	1.15	0.38	1.14	0.41	0.29	0.04	3.70	0.82	0.45	0.17	nd	-	0.97	0.38	0.97	0.27	9.56	1.86
	2014	Winter	0.32	0.03	3.76	1.16	0.14	0.02	0.36	0.06	0.32	0.03	3.90	0.94	0.09	0.00	nd	-	0.49	0.04	0.69	0.03	10.07	2.24
	2014	Spring	0.53	0.14	3.04	1.74	0.45	0.15	0.61	0.20	0.37	0.06	8.93	1.28	0.12	0.04	0.08	0.03	1.83	0.40	1.54	0.43	17.50	3.75
Etiopia MP 3	2013	Winter	0.26	0.13	2.14	0.57	0.15	0.05	1.13	0.32	0.14	0.06	7.12	1.82	0.35	0.10	0.26	0.12	1.55	0.32	2.29	0.36	15.39	3.86
	2013	Spring	0.16	0.01	2.40	0.42	0.05	0.02	0.68	0.08	0.10	0.04	2.27	0.53	0.31	0.06	0.08	0.01	0.80	0.16	0.71	0.13	7.57	1.35
	2014	Winter	0.08	0.03	9.78	2.85	0.09	0.03	0.66	0.03	0.41	0.09	4.52	1.52	0.11	0.01	0.38	0.09	1.70	0.28	3.04	0.58	20.78	0.51
	2014	Spring	0.93	0.30	6.22	0.78	0.08	0.04	0.83	0.13	0.66	0.06	5.37	1.79	0.12	0.03	0.47	0.14	1.92	0.06	2.94	0.08	19.54	2.48
Mutante	2013	Winter	0.16	0.09	0.24	0.10	nd	-	0.35	0.05	0.06	0.01	2.04	0.14	0.03	0.01	0.08	0.03	0.09	0.02	0.46	0.19	3.50	0.04
	2013	Spring	0.71	0.24	0.18	0.06	nd	-	0.91	0.06	0.60	0.23	6.24	1.54	0.24	0.10	0.36	0.04	0.37	0.00	1.67	0.18	11.27	1.57
	2014	Winter	0.34	0.11	1.88	0.51	0.13	0.04	0.67	0.05	0.78	0.31	5.19	1.73	0.12	0.06	0.11	0.03	0.31	0.01	2.66	0.72	12.19	0.94
	2014	Spring	0.33	0.11	2.04	0.55	0.14	0.05	0.76	0.33	0.39	0.08	14.74	4.94	0.15	0.01	0.10	0.01	0.53	0.19	1.77	0.45	20.93	4.78
Trinakria	2013	Winter	0.22	0.06	5.83	0.12	nd	-	1.34	0.13	0.56	0.15	6.46	1.46	0.50	0.04	nd	-	2.98	0.09	2.71	0.42	20.59	1.01
	2013	Spring	0.78	0.44	1.37	0.42	0.10	0.04	0.81	0.29	0.56	0.37	2.63	0.96	0.57	0.27	0.49	0.16	3.63	0.83	3.17	0.61	14.11	4.40
	2014	Winter	0.51	0.17	2.71	0.36	nd	-	0.28	0.04	0.22	0.01	2.11	0.81	0.03	0.01	nd	-	0.57	0.07	0.85	0.07	7.27	1.52
	2014	Spring	0.16	0.05	0.91	0.04	0.06	0.02	0.21	0.01	0.41	0.29	1.73	0.09	0.03	0.01	nd	-	0.41	0.04	0.50	0.02	4.42	0.33

PRO, protocatechuic acid; CAT, catechin; 4-OHB, 4-hydroxybenzoic acid; VAN, vanillic acid; SYR, syringic acid; di-OHB, di-hydroxybenzoic acids; CAF, caffeic acid; p-COU, p-coumaric acid; SIN, sinapic acid; FER, ferulic acid
SD, standard deviation. nd, not detected.

Table 14: Concentration (mean µg/g ± SD) of phenolic acids bound to structures soluble in H_2O, present in four cultivars of durum wheat sown in winter and spring of 2013 and 2014.

Cultivar	year	sowing	**PRO**	SD	**CAT**	SD	**4-OHB**	SD	**VAN**	SD	**SYR**	SD	**di-OHB**	SD	**CAF**	SD	**p-COU**	SD	**SIN**	SD	**FER**	SD	**TOT**	SD
Etiopia ELS	2013	Winter	0.31	0.07	2.72	0.51	1.66	0.36	2.98	0.45	4.21	1.00	36.24	0.95	1.26	0.13	0.12	0.01	24.65	3.26	32.64	2.20	106.78	0.52
	2013	Spring	1.33	0.36	2.81	0.27	1.15	0.04	5.41	0.34	6.45	0.52	57.47	0.70	1.64	0.18	nd	-	24.39	0.06	22.98	0.19	123.63	1.48
	2014	Winter	0.69	0.12	9.90	1.32	1.67	0.17	2.33	0.31	3.76	0.18	50.80	1.94	0.62	0.05	0.20	0.07	17.74	0.00	20.76	1.72	108.48	2.31
	2014	Spring	0.46	0.02	3.74	0.03	1.08	0.17	1.93	0.80	3.00	0.58	48.41	3.02	0.98	0.17	nd	-	23.64	1.37	18.34	1.90	101.57	8.01
Etiopia MP 3	2013	Winter	0.14	0.03	4.04	0.18	3.01	0.38	3.09	0.19	5.01	0.20	54.51	3.40	1.24	0.05	1.16	0.07	23.36	1.47	24.22	1.44	119.77	6.23
	2013	Spring	0.74	0.17	1.66	0.23	1.23	0.17	2.85	0.10	2.98	0.08	57.49	1.40	0.43	0.04	0.84	0.06	20.59	1.26	12.34	1.10	101.16	3.92
	2014	Winter	0.72	0.18	12.92	6.08	2.28	0.81	5.79	0.16	6.13	0.25	65.25	3.66	1.92	0.38	1.10	0.09	21.30	0.42	27.47	1.59	144.88	1.85
	2014	Spring	0.76	0.15	13.33	0.73	2.72	0.91	6.28	0.93	7.76	0.03	71.86	1.64	1.86	0.07	1.51	0.15	25.20	1.20	21.77	0.18	153.04	0.64
Mutante	2013	Winter	0.41	0.10	5.09	1.29	0.87	0.25	1.67	0.70	1.80	0.03	30.34	1.15	0.44	0.21	0.65	0.07	12.67	0.04	16.68	0.43	70.63	0.32
	2013	Spring	0.46	0.13	2.30	0.56	1.01	0.43	3.92	0.37	3.09	0.50	33.49	1.39	1.11	0.17	0.77	0.32	11.45	0.23	17.52	0.31	75.11	0.96
	2014	Winter	0.15	0.05	5.70	0.90	1.80	0.00	1.69	0.76	2.35	0.84	26.47	6.47	0.95	0.32	1.27	0.36	8.91	0.23	16.64	1.95	65.93	11.42
	2014	Spring	2.57	0.24	3.01	0.75	1.33	0.19	1.28	0.33	1.97	0.49	31.20	5.69	0.82	0.27	0.52	0.08	9.02	0.00	12.01	0.84	63.73	8.87
Trinakria	2013	Winter	0.14	0.05	3.65	1.04	0.66	0.07	1.38	0.06	2.63	0.24	15.81	2.95	0.29	0.00	nd	-	15.16	0.51	16.56	0.21	56.28	5.12
	2013	Spring	0.14	0.03	3.67	0.62	0.85	0.10	1.55	0.24	3.58	0.45	22.67	3.71	0.42	0.02	0.75	0.25	15.91	0.43	16.68	0.31	66.23	1.99
	2014	Winter	1.06	0.32	5.12	0.17	1.18	0.20	2.30	0.13	2.33	0.07	24.00	2.90	1.14	0.12	nd	-	10.15	0.13	23.20	0.28	70.46	2.79
	2014	Spring	0.47	0.27	2.90	0.60	1.08	0.52	1.43	0.29	3.07	0.47	13.47	3.94	0.94	0.06	0.13	0.04	6.38	0.04	14.24	0.53	44.12	6.78

PRO, protocatechuic acid; CAT, catechin; 4-OHB, 4-hydroxybenzoic acid; VAN, vanillic acid; SYR, syringic acid; di-OHB, di-hydroxybenzoic acids; CAF, caffeic acid; p-COU, p-coumaric acid; SIN, sinapic acid; FER, ferulic acid
SD, standard deviation. nd, not detected.

Table 15: Concentration (mean µg/g ± SD) of phenolic acids bound to structures soluble in NaOH, present in four cultivars of durum wheat sown in winter and spring of 2013 and 2014.

Cultivar	year	sowing	**PRO**	SD	**CAT**	SD	**4-OHB**	SD	**VAN**	SD	**SYR**	SD	**di-OHB**	SD	**CAF**	SD	**p-COU**	SD	**SIN**	SD	**FER**	SD	**TOT**	SD
Etiopia ELS	2013	Winter	1.44	0.29	5.01	0.56	0.80	0.22	6.02	1.41	0.76	0.22	31.65	4.85	1.65	0.44	2.71	0.90	16.82	2.75	97.89	5.45	164.75	15.61
	2013	Spring	1.41	0.29	1.81	0.59	0.86	0.25	3.02	0.02	2.68	0.44	32.34	1.95	0.66	0.08	nd	-	17.63	0.35	113.24	2.80	173.65	2.28
	2014	Winter	0.85	0.26	2.93	0.04	0.45	0.08	2.35	0.25	2.02	0.40	24.10	3.79	0.46	0.02	nd	-	11.50	0.35	65.14	2.29	109.81	1.85
	2014	Spring	0.94	0.17	1.43	0.52	0.30	0.12	2.89	0.49	2.56	0.16	30.27	3.96	0.52	0.18	nd	-	14.40	3.10	120.36	8.43	173.68	16.10
Etiopia MP 3	2013	Winter	1.81	0.21	5.12	1.18	2.34	0.50	2.58	0.04	1.59	0.03	36.50	4.90	0.59	0.05	2.04	0.03	10.74	0.69	83.21	8.50	146.53	6.27
	2013	Spring	0.98	0.42	3.37	0.78	0.34	0.05	3.31	0.23	2.43	0.86	51.45	3.24	0.69	0.10	2.77	0.20	13.08	2.21	102.26	18.89	180.67	26.53
	2014	Winter	1.79	0.57	5.22	0.75	1.64	0.44	5.20	0.51	2.53	0.06	57.29	5.14	1.12	0.16	1.77	0.19	24.73	2.28	139.26	20.77	240.55	29.62
	2014	Spring	0.82	0.09	2.59	0.76	1.08	0.39	5.30	0.63	7.71	0.06	31.18	2.12	0.89	0.02	2.02	0.02	14.13	1.49	132.45	11.34	198.17	10.07
Mutante	2013	Winter	0.93	0.43	3.20	0.47	0.36	0.07	0.71	0.25	0.78	0.35	24.69	3.76	0.30	0.10	1.42	0.03	7.48	1.44	70.80	9.03	110.67	7.41
	2013	Spring	0.87	0.13	1.21	0.04	0.15	0.03	1.48	0.15	1.39	0.14	18.93	3.29	0.38	0.05	1.93	0.19	5.74	0.06	72.63	2.23	104.70	6.06
	2014	Winter	1.25	0.05	2.07	0.57	1.04	0.01	3.28	1.09	2.93	0.74	59.91	5.50	0.27	0.02	0.59	0.22	6.45	0.16	85.42	11.33	163.21	18.14
	2014	Spring	1.20	0.17	2.31	0.39	1.25	0.06	3.13	1.04	3.26	0.84	29.38	5.88	0.17	0.02	1.08	0.42	8.86	1.38	105.38	7.12	156.01	5.45
Trinakria	2013	Winter	0.46	0.16	4.78	0.83	0.44	0.21	1.46	0.48	1.51	0.03	19.27	1.05	0.36	0.04	nd	-	13.97	1.01	64.67	3.65	106.93	7.41
	2013	Spring	0.70	0.17	4.74	0.57	0.88	0.21	1.90	0.32	6.46	1.61	30.06	4.94	0.47	0.02	0.33	0.11	16.80	0.00	66.43	4.74	128.77	0.96
	2014	Winter	0.46	0.16	2.59	0.64	0.25	0.05	1.93	0.35	1.50	0.33	16.03	4.32	0.68	0.10	nd	-	7.84	1.84	118.02	13.24	149.29	20.60
	2014	Spring	1.33	0.05	3.52	0.90	0.30	0.05	1.48	0.07	1.14	0.14	53.05	4.58	0.27	0.05	nd	-	6.14	0.69	85.46	6.43	152.68	11.08

PRO, protocatechuic acid; CAT, catechin; 4-OHB, 4-hydroxybenzoic acid; VAN, vanillic acid; SYR, syringic acid; di-OHB, di-hydroxybenzoic acids; CAF, caffeic acid; p-COU, p-coumaric acid; SIN, sinapic acid; FER, ferulic acid
SD, standard deviation. nd, not detected.

Table 16: Mean concentrations ± SD of resistant and non-resistant starch in grams of glucose (Glc) per 100g of durum wheat flour and grams of starch per 100g of durum wheat flour.

			g Glc/100 g flour[a]				g Starch/100g flour			
Cultivar	Year	Season	RS mean	SD	NRS mean	SD	RS mean	SD	NRS mean	SD
Etiopia ELS	2013	Winter	0.24	0.19	27.79	8.68	0.22	0.17	25.01	7.81
	2013	Spring	0.11	0.08	27.41	5.25	0.10	0.07	24.67	4.72
	2014	Winter	0.52	0.07	38.11	3.24	0.47	0.07	34.30	2.92
	2014	Spring	0.47	0.04	41.08	5.01	0.43	0.04	36.97	4.51
Etiopia MP 3	2013	Winter	0.39	0.27	29.89	2.02	0.35	0.25	26.90	1.81
	2013	Spring	0.12	0.08	31.33	9.74	0.11	0.07	28.20	8.77
	2014	Winter	0.15	0.03	38.93	6.82	0.14	0.02	35.04	6.14
	2014	Spring	0.21	0.01	21.55	4.98	0.19	0.01	19.40	4.48
Mutante	2013	Winter	0.31	0.18	29.21	1.29	0.28	0.17	26.29	1.16
	2013	Spring	0.10	0.01	25.43	4.01	0.09	0.01	22.89	3.61
	2014	Winter	0.15	0.02	26.27	5.08	0.13	0.02	23.65	4.58
	2014	Spring	0.20	0.08	26.69	0.91	0.18	0.07	24.02	0.82
Trinakria	2013	Winter	0.14	0.03	27.28	2.73	0.13	0.02	24.55	2.46
	2013	Spring	0.12	0.00	26.86	4.82	0.10	0.00	24.17	4.34
	2014	Winter	0.30	0.19	26.57	4.93	0.27	0.17	23.91	4.44
	2014	Spring	0.04	0.02	26.60	0.07	0.03	0.02	23.94	0.06

[a] Data used in the statistical analysis
RS, resistant starch; NRS, non-resistant starch